SOY ISOFLAVONES AND SWINE HEALTH

BY

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DISSERTATION

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ABSTRACT

Due to increased regulation and mounting consumer concerns, utilization of in-feed antibiotics to promote productivity is declining in modern U.S. livestock production systems. For that reason, there is increased interest and opportunities to identify novel feed additives or value-added feed ingredients that may function as nutritional interventions to improve the health and productivity of our livestock species. Previously, swine nutrition research has found that pigs experiencing high levels of inflammatory stress performed better when fed greater amounts of soybean meal in the diet though it was unclear which component of this soy protein source was conferring these benefits. Among potential components, soy-derived isoflavones show promise as a nutritional intervention against a pathogenic challenge due to their antiviral and anti-inflammatory properties. For swine in particular, there are a number of pathogenic challenges a pig may face throughout its productive life, with immediately post-weaning being a particularly vulnerable phase. A series of three experiments was conducted in order to investigate the effect of dietary soy isoflavones on the growth and immune responses of weaned pigs to a common and pervasive viral pathogen affecting U.S. swine herds, porcine reproductive and respiratory syndrome virus (PRRSV). In the first experiment we sought to determine if soy isoflavones fed at concentrations typically found in standard commercial swine diets would demonstrate protective effects against an acute PRRSV infection in weaned pigs in the presence of two soy protein sources (soy protein concentrate [SPC] and enzyme-treated soybean meal [ETSBM]). While isoflavones minimally impacted growth performance during this acute challenge, they modified the cellular immune response to PRRSV infection by reducing PRRSV-induced band neutrophilia and improving cytotoxic-to-helper T-cell ratios with only a few interactions occurring between soy protein type and isoflavones supplementation across all remaining

immune measures collected. These results suggested that isoflavones, regardless of protein source, may influence the activation of adaptive immune system and may facilitate recovery from and clearance of PRRSV infections. To extend those findings, we next sought to determine if soy isoflavones fed at the same level would demonstrate protective effects against both the acute and recovery phases of a PRRSV infection in weaned pigs and if those effects would benefit the growth performance across the entire growth period from weaning to market. Similar to the first experiment, soy isoflavones elicited inconsistent effects on growth performance, but again influenced cellular and global immune responses to PRRSV infection. Isoflavone supplementation increased neutrophil cell counts and the relative proportion of memory T-cells, and decreased the time to full PRRSV clearance from oral fluids. However, the most notable effect isoflavones had in this experiment was reducing pathogen-associated mortality by ~50%, which may have significant economic implications for producers. These findings suggested that isoflavones enable sufficient immune responses needed to reduce mortality in this infection model, but it remained unclear if isoflavones were eliciting these effects directly or if they functioned through an indirect route such as alteration of the gastrointestinal microbiome. For that reason, a third experiment was conducted to evaluate how the ingestion of soy isoflavones in the context of a PRRSV-infection affected the composition of the gastrointestinal microbiome across the entire growth period. Across all time points measured, microbiome analyses indicated that the relative abundance of only a few bacterial populations were directly affected by isoflavones and differences detected were mainly due to PRRSV infection alone. These findings imply that previously observed performance benefits conferred by isoflavones within this PRRSV infection model were not likely due to changes in microbiome composition. Overall, this research demonstrates that isoflavones do not negatively impact the productivity of PRRSV-

challenged pigs and may confer additional health benefits through supporting systemic immune responses in the face of a pathogenic challenge, though more research in robust live animal models is merited.

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CHAPTER 1: INTRODUCTION

As modern animal agriculture continues to progress and evolve, new challenges are regularly presented to livestock producers. Increased consumer concerns about the risks of antibiotic resistance driven by normal practices in large scale livestock production have led to increased regulation and potential incentives against utilizing in-feed antibiotics. With the passing of the U.S. Veterinary Feed Directed in 2017, a government mandate seeking to cease long-term applications of in-feed antibiotics as growth promotants, livestock producers and nutritionists are tasked with the transition of feeding livestock with greater limitations on the use of sub-therapeutic antibiotic interventions (FDA, 2015). For that reason, there has been increased interest in the development of feed additives or value-added feed ingredients and their potential to mitigate negative performance responses to disease in today's livestock herds.

Regarding specifically pork production, among feed ingredients commonly used soybean meal (**SBM**) has been associated with better performance in growing pigs under inflammatory conditions caused by disease. In a novel observation by Boyd et al. in 2010, pigs that experienced an unplanned pathogenic challenge grew better by industry standard measures when they received higher levels of SBM in the diet than their experimental counterparts that received crystalline amino acid products. This has led swine nutrition researchers to question how these benefits were derived from this whole soy protein source. Was it simply due to increased amino acid availability in the diet or were there other naturally occurring components of soybeans driving these effects?

Focusing on the latter of those two possibilities, one such component that demonstrates biological potential to mediate the physiological response to disease are soy isoflavones. While more commonly recognized in human health applications for their structural similarity to

endogenous estrogens, soy isoflavones have been shown to possess anti-inflammatory and anti-viral activity in both *in vitro* and *in vivo* experimental models. These activities include direct modification of cell signaling pathways, interruption of viral replication or spread, and alterations of key immune responses to pathogens (Andres et al., 2007; Dia et al., 2008; Andres et al., 2009; Smith and Dilger, 2018). While a majority of these effects have been demonstrated in human disease models, there has been evidence that isoflavones have potential to mitigate the response to swine-specific pathogens, particularly those causing respiratory or gastrointestinal disease (Smith and Dilger, 2018).

Among pathogens affecting U.S. swine herds today, porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most pervasive and economically detrimental pathogens facing pork producers today (Holtkamp et al., 2013). For that reason, it is an extremely relevant disease model when determining the efficacy of novel health-promoting feed additives. Against PRRSV, there is evidence that individually supplemented soy isoflavones (Greiner et al., 2001a; Greiner et al., 2001b) and diets containing higher levels of soy isoflavones from increased SBM inclusion (Rochell et al., 2015) both improved both growth performance and certain immune responses to PRRSV infection. However, at the completion of these studies it is was still unclear whether isoflavones, if provided at levels and ratios that are typical of standard commercial swine diets, would still provide performance benefits to pigs infected with PRRSV, particularly if the protein content of the diet is controlled. More so, if protein levels are controlled is it possible to determine if soy isoflavones are causing these benefits directly or is their biological activity through an indirect mechanism such as modification of their metabolism through shifts in gastrointestinal microbiome composition.

For those reasons, the overarching objective of the current research described in the following document was to determine if dietary soy isoflavones provide performance benefits to growing pigs faced with a pathogenic challenge and, if they do confer benefits, attempt to identify a potential biological mechanism. This objective was approached by three different experimental aims:

Aim 1) Determine if soy-derived isoflavones fed to weanling pigs would demonstrate protective effects during the acute phase of a PRRSV-infection in the presence of two different soy protein sources

Aim 2) Determine if soy-derived isoflavones fed to weanling pigs would demonstrate protective effects during both acute and recovery phases of a PRRSV-infection in the presence of a single soy protein source and how those effects, if present, influence performance over the entire growth period from weaning to market

Aim 3) Evaluate how consumption of soy-derived isoflavones by PRRSV-infected pigs affects the longitudinal composition of the gastrointestinal microbiome throughout the entire growth period from weaning to market

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CHAPTER 2: LITERATURE REVIEW

Role of Soy Protein in Swine Production[1]

Soybeans have a rich history of use as valuable protein and fat sources in both human and animal diets. By 1982, soybean meal (**SBM**) became the most commonly used protein source in livestock diets in the United States, over other popular sources including cottonseed meal and fish meal by a wide margin (Shurtleff and Aoyagi, 2007). According to the USDA National Agricultural Statistics Service, ~30,035 metric tons of soybeans were used domestically for livestock feed in 2016 alone (USDA-NASS, 2017). SBM is created through the collection of soy oil, a process by which soybeans are cleaned, cracked, de-hulled, and heat-treated before being crushed into flakes. Flakes are then washed with a volatile solvent, such as hexane, to extract crude oil, thereby leaving behind a high-quality protein ingredient. Oil-extracted flakes (i.e., defatted soybeans) undergo an additional heating step (i.e., toasting) to remove residual solvent and, in many cases, are mixed back in with soybean hulls and ground to form SBM, which can be fed to livestock. Toasting soybean flakes towards the end of processing also contributes to the removal of certain antinutritional factors, mainly urease and lipoxygenase enzymes (Shurtleff and Aoyagi, 2016). SBM is the most commonly used plant-based protein source in swine diets throughout the post-weaning growth period in the United States. Its popularity stems from a relatively high digestible protein fraction, high lysine content to complement dietary corn inclusion, and the ability to extract antinutritional factors through processing (Pettigrew et al., 2017). And while SBM remains a widely used protein source in swine diets, advancements in soybean processing techniques and applications have resulted in development of value-added feedstuffs. Soy protein concentrate (**SPC**) is produced from defatted soybean flakes, as is SBM,

[1]Sections adapted from Smith, B. N. and R. N. Dilger. 2018. Immunomodulatory potential of dietary soybean-derived isoflavones and saponins in pigs. J. Anim. Sci. 96:1288-1304. doi: 10.1093/jas/sky036.

but undergoes additional processing steps to remove the soluble carbohydrate fraction. Two methods are employed for this step, extraction or enzymatic digestion, with extraction being more common. Extraction is the process of solubilizing carbohydrates with an aqueous alcohol solution, removing not only vulnerable soluble carbohydrates but also residual antinutritional factors including oligosaccharides, as well as estrogenic and antigenic factors (Peisker, 2001). Soy protein isolate (**SPI**) is essentially the same as SPC, with the residual non-soluble carbohydrate fraction left behind after alcohol extraction removed. Further processing of soybeans into SPC and SPI results in products with a higher protein composition compared with SBM (65% to 90% and ≥ 90% vs. 40% to 50% for SPC, SPI, and SBM, respectively; Shurtleff and Aoyagi, 2016).

Despite the popularity of using SBM in swine diets, among the different growth phases of current production systems, diets of early-weaned pigs traditionally contain minimal amounts of soy. This practice of low SBM inclusion is largely driven by immaturity of the weanling pig's digestive capacity and effectiveness, in addition to presence of dietary antinutritional factors to which young pigs are more sensitive. However, developments in soy processing (described above) and treatment of SBM-containing diets have made feeding soy protein to early-weaned pigs more appropriate. In addition to removal of antinutritional factors through production of SPC and SPI, microbial fermentation of soy feedstuffs can be utilized during this sensitive period for weaned pigs. Early-weaned pigs provided fermented liquid diets during the initial post-weaning period typically show improved growth performance (~22% improvement for average daily gain) and reduced disease level with respect to enteric pathogens such as *Escherichia coli* and *Salmonella* due to unfavorable acidic gastrointestinal environment (Stein, 2002). Early-weaned pigs that receive microbial-fermented soy protein sources in more traditional dry diets

similarly show improved growth performance, increased nutrient digestibility, and, under certain experimental conditions, reduced diarrhea scores, an important indicator of improved disease status in young pigs (Cho et al., 2007; Kim et al., 2007; Kim et al., 2010; Zhang et al., 2013).

Soy Protein and Swine Health[2]

In addition to the challenges that dietary transitions and protein source can pose to young pigs, the early weaning period in general presents a myriad of stressors to the growing pig. The stress of weaning, mixing, and movement to an unfamiliar environment all contribute to decreased performance during this time of transition and also puts young pigs at greater risk of succumbing to unfamiliar pathogens. Interestingly, there is evidence that under inflammatory conditions, diets high in SBM improved average daily gain (**ADG**), feed conversion ratio (**FCR**), and whole-body and carcass lean tissue accretion in pigs. This is in contrast to utilizing crystalline amino acids (**AA**) in diets of growing pigs, which has been reported to improve growth performance of healthy pigs when compared with intact protein sources. The practical implication is that crystalline AA may be economical and effective to feed to healthy pigs, but SBM should be utilized in pigs or herds under high inflammatory stress. The benefits of SBM in this context are confounded, however, with crude protein (**CP**) content of the diet. Benefits could be due to anti-inflammatory properties of compounds such as isoflavones or saponins or could be due to increased availability of AA in the diet to support immune tissues (Boyd et al., 2010). While several groups have attempted to elucidate what particular component of the soybean confers additional benefit to pigs facing strong inflammatory stressors, our group found particular interest in investigating soy isoflavones due to their range of bioactivity.

[2]Sections adapted from Smith, B. N. and R. N. Dilger. 2018. Immunomodulatory potential of dietary soybean-derived isoflavones and saponins in pigs. J. Anim. Sci. 96:1288-1304. doi: 10.1093/jas/sky036.

Soy Isoflavones[3]

Structure and Metabolism

Isoflavones (**ISF**) are naturally occurring flavonoid compounds found at high concentrations in the soybean plant. They are characterized as phytoestrogens due to their structural similarities to 17β-estradiol (**E2**), though their biological action is not exclusively estrogenic in nature. Genistein and daidzein comprise the majority of isoflavones found in soybeans and are the subject of most ongoing research. Isoflavones exist in the plant as non-bioavailable glycoside forms (e.g., genistin and daidzin). For isoflavones to be absorbed and biologically available, they must be hydrolyzed within the digestive tract by host-derived or microbial β-glycosidases to their aglycone forms (e.g., genistein and daidzein). β-Glycosidases are present as brush border enzymes in the small intestine, but are more prevalent as microbial enzymes in the hindgut of the gastrointestinal tract of monogastric species like the pig (Cassidy et al., 2006). Genistein and daidzein are either directly absorbed in the intestine or undergo further metabolism via hydrogenation to other bioactive compounds, including equol, O-desmethylangolensin (**ODMA**), 5,7,4-rihydroxyisoflavan, 4,7,4-trihydroxyflavan, dihydrodiadzein (**DHD**), dihydrogenistein (**DHG**), and others. These hydrogenation reactions are non-specific, resulting in non-uniform mixtures of these compounds (Chang et al., 1995; Nakatsu et al., 2014). In humans, isoflavones absorbed from the gastrointestinal tract are present in plasma for 5 to 8 h post-prandial (Cassidy et al., 2006). Only a small fraction (<2%) of isoflavones enter circulation with a majority of soy isoflavones being directly metabolized by enterocytes, which may indicate that isoflavones exert maximum functionality locally in the gastrointestinal tract (Masilamani et al., 2012). Similar plasma concentrations of isoflavones

[3]Sections adapted from Smith, B. N. and R. N. Dilger. 2018. Immunomodulatory potential of dietary soybean-derived isoflavones and saponins in pigs. J. Anim. Sci. 96:1288-1304. doi: 10.1093/jas/sky036.

have been observed in humans and pigs receiving soy-containing diets, though the proportions of different isoflavones and their metabolites excreted in urine differ, suggesting that individual isoflavone biological activity and cellular consumption may also differ between species (Gu et al., 2006). In addition to differences in metabolism of dietary isoflavones, the bioavailability of isoflavones in a given feedstuff can vary significantly.

Factors Affecting Bioavailability of Soy Isoflavones

There are several factors that affect bioavailability of dietary isoflavones, the first of which influence isoflavone content in the soybean plant itself. Factors such as plant variety, location, and growing conditions can all affect isoflavone contents of any given yield and variations of these factors have been considered for some time. Studies have demonstrated that total glycoside isoflavone content of a single soybean plant variety can vary as much as 200 mg/g when the same variety is grown at different regional locations. On a year-to-year basis, variation in isoflavone concentrations of up to 50% has been reported within the same growing location (Eldridge and Kwolek, 1983). Additionally, differing patterns of individual isoflavone distribution among soybean crops maintained in different countries (including United States, Brazil, Argentina, and Japan) suggest that plant genetics have an effect on isoflavone development and metabolism (Wang and Murphy, 1994; Goerke et al., 2012). Although plant variation contributes to total isoflavone content within the raw soybean, soybean processing has an even greater influence on final product concentrations.

Processing techniques, such as the ones described in the previous sections, can greatly affect the final isoflavone content in soy products and, subsequently, their bioavailability. Although most of the data available for soy processing effects are from products for human consumption, the principles remain the same for livestock feedstuffs. Roasted soy products,

including SBM, contain the highest bioavailable content of glycoside isoflavones, whereas SPC and SPI contain the lowest concentrations (Erdman et al., 2004). This difference is due to ingredient processing methods, and while the defatting process of full fat soybeans in the generation of SBM does not affect isoflavone concentrations, aqueous alcohol extraction utilized to generate SPC removes >95% of total isoflavones (Kuhn et al., 2004). Manufacturing processes for SPI, a product mostly devoid of fats and carbohydrates, result in over a 50% reduction in isoflavone content due to the alkaline extraction methods used. A summary of typical isoflavone concentrations in SBM, SPC, and SPI can be found in **Table 2.1**. Thus, isoflavones are lost into the alkaline-insoluble fraction, which is typically discarded in the processing procedure. Alternatively fermented soy products contain a higher bioavailable content due to microbial enzyme hydrolysis of isoflavones from their glycoside to aglycone forms (Wang and Murphy, 1996).

Food matrix may also affect isoflavone metabolism kinetics. In a human trial, liquid diets resulted in faster isoflavone absorption rates and higher peak plasma concentrations compared with solid diets (Cassidy et al., 2006). As mentioned previously, fermented liquid feeding and fermented SBM have been suggested to improve nursery pig performance. Although no confirmatory evidence exists, it may be possible that the isoflavone fraction of diets containing fermented soy products may be more bioavailable, resulting in faster absorption and delivery to target cells, thereby contributing to beneficial performance effects.

Estrogenic Properties of Soy Isoflavones

Due to their structural similarities to 17β-estradiol (**E2**), isoflavones have the ability to act as weak agonists or antagonists for endogenous estrogen, depending on the concentrations present at the cellular level. Isoflavones, especially genistein, preferentially bind estrogen

receptor-β (**ER-β**) vs. estrogen receptor-α (**ER-α**). Estrogen receptor-β is expressed on a variety of cell types, including uterine epithelial cells and immune cells such as blood monocytes and tissue macrophages, and is the most prevalent estrogen receptor found within the gastrointestinal tract. Although isoflavones may act as estrogen receptor modulators, their potency is 1,000-fold lower than that of endogenous estrogen, so it is unlikely physiological effects induced by isoflavones, especially in regard to response to disease, is through estrogenic activity alone (Andres et al., 2009). There is evidence that ER-β modulates ER-α activity during uterine development in mice via anti-proliferative effects, which could indicate that circulating isoflavones may influence reproductive development (Weihua et al., 2000). In pigs, there is less information available on the effects of soy isoflavones on the reproductive tract of intact females. One study evaluated the effect of oral genistein administration on hormonal patterns of gilts during estrus and following artificial insemination (**AI**). Gilts exposed to genistein via oral administration (1 mg/kg body weight [**BW**] twice daily) demonstrated increased plasma concentrations of oxytocin and prostaglandin E2 (**PGE2**) and a more frequent pulsatile pattern in prostaglandin F2-α (**PGF2-α**) concentrations around administration of AI. Alternatively, genistein decreased plasma luteinizing hormone (**LH**) concentrations after AI was administered compared with control gilts. These results suggest that hormonal release may be altered by soy isoflavones, likely through interactions with estrogenic receptors, but the impact of these alterations on gilt reproductive performance is not well understood (Norrby et al., 2011).

Anti-Inflammatory and Antioxidant Properties of Soy Isoflavones

Soy isoflavones possess both anti-inflammatory and antioxidant activities, which has generated a lot of attention for applications in both humans and animals. These activities are mainly observed through isoflavones' inhibitory effects on tyrosine-specific protein kinases and

nuclear factor-κB (**NF-κB**) transcription. Tyrosine-specific protein kinases act as regulators for a broad spectrum of cellular functions. These protein kinases respond to several cell-signaling molecules including growth factors (i.e., epithelial growth factor, insulin-like growth factor-1) and cytokines, regulating cell proliferation and transformation properties (Akiyama et al., 1987). They are also the target of several virus types and are likely the main mechanism of action of reducing viral infectivity, though several effects have been reported and thus the reduction in infectivity is likely a combination of several pathways (Andres et al., 2009). For the purpose of this review, effects of isoflavones on the antigen-specific immune response, anti-oxidative cellular pathways, and viral infectivity will be discussed in more detail.

Soy isoflavones have been found to suppress antigen-specific immune responses in addition to exhibiting broad anti-inflammatory activities. Antigen-specific immune responses are highly involved in sensitization to dietary antigens, establishment of allergic responses, and autoimmune reactions. Of the antigen-specific immunomodulatory effects mediated by isoflavones, interactions with dendritic cells seem to be involved in the mechanism of action. Mice fed genistein- and daidzein-enriched diets that were orally sensitized and challenged with peanut-derived proteins exhibited reduced anaphylactic symptoms, with 35% to 40% having no or significantly less severe symptoms compared with control mice, and 25% to 30% decrease in mast cell degranulation. Isoflavones also decreased the synthesis of peanut-specific IgE and IgG2a humoral antibodies, which suggests that these isoflavones may also mediate B-cell activity. However, since these effects were not maintained when peanut-derived proteins were injected intra-peritoneally, the authors suggested that diet-derived isoflavones may be more likely to elicit local inhibition of dendritic cells, such that supra-physiological concentrations would be necessary for total systemic suppression of allergic sensitization (Masilamani et al.,

2011). In an *in vivo* collagen-induced arthritis model, representative of an autoimmune reaction, mice injected subcutaneously with genistein had suppressed delayed-type hypersensitivity reactions to oxazolone and granulocyte-mediated responses compared with control animals. The suppression of cell-mediated responses was likely not through estrogen-receptor binding since co-injection with ICI 182,780, an estrogen-receptor antagonist, did not prevent the inhibitory action of genistein. More likely, the mechanism of action for cell-mediated immune inhibition is through protein kinase inhibition in B-cells. This inhibition of protein kinase activity in B-cells results in decreased antigen processing and subsequent antibody production (Verdrengh et al., 2003). Although these findings are relevant, especially for human medical researchers, the antioxidant potential for isoflavones may be of stronger interest for animal nutritionists.

The antioxidant activity of isoflavones is mainly mediated by their inhibition of NF-κB, a protein complex that controls the transcription of many pro-inflammatory genes. Based on *in vitro* findings, anti-oxidative effects via NF-κB inhibition include reduction in inducible nitric oxide synthase (**iNOS**) expression, nitric oxide (**NO**) production, cyclooxygenase-2 (**COX-2**) expression, and PGE2 production (Dia et al., 2008). In lipopolysaccharide (**LPS**)-stimulated RAW 264.7 macrophages, genistein decreased NO production in a dose-dependent manner (IC_{50} = 69.4 μM) without adverse effects on cell viability, suggesting that genistein disrupted the induction of iNOS expression and production of downstream products (e.g., nitric oxide). In a similar experiment, 50 and 100 μM concentrations of genistein-reduced accumulation of thiobarbituric acid reactive substance (**TBARS**), an indicator of lipid oxidation, increased concentrations of glutathione, an important antioxidant, and increased activities of anti-oxidative enzymes superoxide dismutase and catalase (Choi et al., 2003). When provided individually, genistein and daidzein decreased NO production and iNOS expression in a dose-dependent

manner (genistein at 10 μM vs. daidzein at 100 μM), decreased PGE2 production, and decreased COX-2 expression (genistein at 100 μM vs. daidzein at 300 μM). However, the magnitude of inhibitory effects was highest when a mixture of soy isoflavone glycosides was used, suggesting interactions among the isoflavone forms (Dia et al., 2008). Similarly, in LPS-activated murine J774 macrophages, genistein and daidzein were shown to elicit moderate inhibitory effects via NF-κB on iNOS expression (57% to 72% inhibition, IC_{50} for genistein and daidzein was ~30 and ~70 μM, respectively) (Hämäläinen et al., 2007). Many of these pathways are employed directly during bacterial and viral insults, but there is additional evidence that isoflavones may also affect specific viral infectious pathways that may lead to reduced viral infectivity.

Anti-viral Properties of Soy Isoflavones

Building upon the backdrop of broad immunomodulatory actions of isoflavones, soy isoflavones may benefit the immune response under viral-challenged conditions. Although many of the findings discussed in this section were gathered from studies involving *in vitro* human cell lines or mice, many of the viruses investigated are some of the same classes of viruses commonly present in the swine industry.

Rotavirus, a virus that causes acute enteritis in neonates of several mammalian species, is one such virus whose infectivity is modulated by soy isoflavones. In a study by Andres et al., genistein and daidzein inhibited infectivity of human rotavirus in cultured macrophages (MA-104 cell line). Action by genistein alone reduced rotaviral infectivity by 33% to 62% and a mixture of isoflavones resulted in a reduction of 66% to 72%. Mixtures not containing genistein lost antiviral activity, indicating that genistein is likely the most active isoflavone in mediating rotaviral inhibition. This particular inhibition was thought to be through inhibition of tyrosine-specific protein kinase-induced activation of $\alpha 2$-$\beta 1$ binding of the virion (Andres et al., 2007).

In an *in vitro* assay evaluating flavonoid modulation on infectivity of another common virus, herpes simplex virus types-1 and -2 (**HSV-1** and **HSV-2**), genistein exhibited high inhibitory activity against cytopathic effects (**CPE**; i.e., structural changes caused by viral infection in host cells that result in host cell death). These effects were considered moderate (50% to 80% CPE inhibition at 5 μM concentrations), but were observed for both viral types (Lyu et al., 2005). Reductions in herpes virus infectivity by genistein were also demonstrated for *Bovine herpesvirus* (**BHV-1**), where genistein reduced BHV-1 viral replication by 90% by 18 h post-inoculation when infected cells were treated with 25 μM at 0 and 12 h (Akula et al., 2002).

While these *in vitro* studies confirm that soy isoflavones demonstrate suppressive activity against certain viral pathogens, regarding their potential role in swine health, we are specifically interested in their potential to modulate the response to common swine diseases in the live animal. For that reason, the focus of the current research described in the following data chapters has been how soy isoflavones modulate the clinical response of growing pigs to one of the most common viral pathogens facing U.S. swine production, porcine reproductive and respiratory syndrome virus.

Porcine Reproductive and Respiratory Syndrome Virus: The Porcine Immune System and Its Response to PRRSV

Porcine Reproductive and Respiratory Syndrome Virus

Porcine reproductive and respiratory syndrome virus (**PRRSV**) is a positive-stranded, enveloped RNA virus of the family *Arteriviridae*. It is species-specific (i.e. only affecting swine) and, due to it being an RNA virus, is highly variable. Infection causes abortions, stillborn piglets, and other reproduction-related failures in sows, increased pre-weaning death, acute respiratory disease, anorexia, and reduced growth performance in nursing and growing pigs, and acute respiratory disease, anorexia, and reduction of semen quality in boars (Tousignant et al., 2015). It

is currently one of the most economically important diseases affecting swine, resulting in production losses within breeding and growing swine herds to the tune of hundreds of millions of dollars annually (Holtkamp et al., 2013; Schweer et al., 2017). Of the contributing factors to these losses, ease of transmission between animals, heterogenic properties of the virus itself, and inconsistent and ineffective immune responses to PRRSV by individual pigs resulting in failure to development herd immunity are among the most significant.

Pigs can be exposed to PRRSV through a number of routes. The virus is transmitted through virtually all bodily secretions: saliva, nasal secretions, urine, semen, and feces. Gilts and sows that are exposed during late gestation (i.e., third trimester) can transfer the virus transplacentally to fetuses or through colostrum and milk. Not all routes are as equally susceptible to establishment of infection, but the most common susceptible routes of horizontal transmission are as follows: parenteral (i.e., breaks in the skin barrier) or intramuscular, intranasal, and oral. Airborne transmission (in addition to arthropod-borne transmission) also contributes to horizontal transfer, but is difficult to document under field conditions (Zimmerman, 2006). PRRSV preferentially targets cells of monocyte/macrophage lineage, specifically porcine alveolar macrophages (**PAM**) of the respiratory tract which express macrophage-specific scavenger receptor CD163 (Kim et al., 2006; Welch and Calvert, 2010). Due to its tropism being an important cell for both the innate and adaptive immune response, the virus's ability to manipulate the host response to PRRSV poses a unique challenge to swine producers.

Introduction to the Porcine Immune System

In order to fully understand how PRRSV manipulates the host immune response, it's helpful to understand how the immune system functions under normal conditions. The immune

system is comprised of two functional sub-systems: the innate immune system and the adaptive immune system. While these two systems work in concert during active immune responses, often performing effector functions that collectively enable pathogen clearance, it is easier to understand their role in the immune response if they are described separately. The innate immune system is often described as the "first line of defense" against a pathogen; it is comprised of physical epithelial and mucosal barriers, soluble proteins (i.e., complement), and effector cells that respond predictably to general, highly conserved pathogen-associated signals (i.e., pathogen-associated molecular patterns or **PAMP**) (Murphy and Weaver, 2017). In the event that physical and chemical barriers of the innate immune system are breached, due to their ability to recognize these general signals, innate effectors cells are able to rapidly respond to the presence of a wide variety of pathogens. There are several cell types associated with the mammalian innate immune system including granulocytes (neutrophils, eosinophils, and basophils), macrophages, dendritic cells, and natural killer cells (**NK**) (Murphy and Weaver, 2017). Granulocytes are categorized by their cytoplasmic granules and all demonstrate some phagocytic activity, though neutrophils exhibit the greatest phagocytic activity among them. Once these cells recognize a PAMP, they are rapidly activated to phagocytose pathogens and secrete signaling molecules called cytokines to help recruit additional immune cells to the site of infection. This response is extremely rapid, often taking place within minutes of a pathogen being detected (Murphy and Weaver, 2017).

In addition to granulocytes, macrophages function as the major phagocyte population of the innate immune system and reside in most peripheral tissues normally. They can develop from bone marrow progenitor cells or from activated circulating monocytes during infections or periods of inflammation. In addition to their phagocytic activity, macrophages also secrete

cytokines and function as antigen presenting cells (**APC**) alongside dendritic cells, a third class of phagocytic cells that reside in lymphoid and peripheral tissues, and B-cells, a cellular member of the adaptive immune system (Murphy and Weaver, 2017). The main function of antigen presentation is the activation of adaptive immune cells, which is a key way the two systems integrate together.

The adaptive immune system, unlike the innate immune system, responds less rapidly to primary apathogenic insult but with a much greater specificity for a particular pathogen. This not only helps facilitates more targeted clearance of a pathogen, it also results in the development of immunological memory or the ability to prevent an infection by the same pathogen from occurring again in the future. The adaptive immune system is comprised of two main cell types: T-cells, which drive cell-mediated immunity, and B-cells, which drive humoral immunity. In mammalian species, mature B-cells and T-cells reside in lymphoid tissues such as lymph nodes and undergo activation once presented with an antigen by an APC. For T-cells, this activation involves recognition of presented antigen on major histocompatibility (**MHC**) class I or II molecules. T-cells vary in their effector function, differentiated by cluster of differentiation (**CD**) cell surface markers. Two key effector T-cell types are cytotoxic T-cells and helper T-cells. Cytotoxic T-cells (**CTL**; positive for cell-surface marker CD8) recognize antigens presented on MHC class I molecules and their main function is targeted cell death of infected cells. Helper T-cells (positive for cell-surface marker CD4) recognize antigens presented on MHC class II molecules and their main function is the coordination of immune response and the activation of B-cells. B-cells are activated by recognition of an antigen by surface immunoglobulin receptors. Once these receptors recognize and bind their antigen, they then present the antigen in MHC class II molecules in order to bind helper T-cells, which complete the activation process. Fully

activated B-cells will proliferate and mature into antibody secreting cells (i.e., plasma cells), which further support pathogen clearance and immunological memory (Murphy and Weaver, 2017).

Understanding the general components and dynamics of the immune system, the normal immune response to viral pathogens specifically may be described in more detail. Being that they are typically categorized as intracellular pathogens, in the event of a viral infection virions enter a cell where it recognized by intracellular pathogen recognition receptors (**PRR**), endosomal Toll-like receptors (**TLR**) and cytoplasmic PRR. Regarding TLR specifically, the TLR responsible for viral recognition include TLR3, TLR7, TLR8, and TLR9, which are highly expressed in APC of the innate immune system such as macrophages and dendritic cells. Once the receptors recognize the virus, they activate several cellular pathways that result in the production of inflammatory cytokines, chemotactic factors (i.e., chemokines, proteins similar to cytokines that help direct other immune cells to the site of infection), and antiviral cytokines interferon-α (**IFN-$\boldsymbol{\alpha}$**) and interferon-β (**IFN-$\boldsymbol{\beta}$**) (Murphy and Weaver, 2017). Of these actions, the production of type I interferons IFN-α and IFN-β result in direct anti-viral effects including induced resistance to viral replication, increased MHC class I expression and antigen presentation to CTL, activation of dendritic cells and macrophages, activation of NK cells to kill virus-infected cells, and influence additional cytokine and chemokine secretion to recruit lymphocytes to the site of infection (Bogdan, 2000; Murphy and Weaver, 2017). The activation of these anti-viral pathways is key for the recognition and clearance of viral infections; if compromised, as it is in active PRRSV infections, then viral infections are able to persist, resulting in greater viral replication and spread.

The Porcine Immune Response to PRRSV – The Innate Response

As described, upon initial exposure to a viral insult tissue-resident innate immune cells macrophages and dendritic cells produce anti-viral, pro-inflammatory type I interferons, IFN-α and IFN-β (Loving et al., 2015). PRRSV dampens the type I interferon response, with peak IFN-α titers being minimally 1,000-fold lower than that observed during other common swine respiratory viruses (e.g., swine influenza virus, **SIV**, and porcine respiratory circovirus, **PRCV**). It also appears to reduce the production of other key pro-inflammatory cytokines such as tumor necrosis factor-α (**TNF-α**) (van Reeth et al., 1999). It has been suggested that this inhibition is likely through PRRSV mediated down-regulation of TLR3, TLR7, and TLR8, which are important PRR for viruses. Of these, TLR3 appears particularly important for preventing PRRSV replication, demonstrating increased PRRSV replication and delayed IFN-α expression when its activation is reduced (Sang et al., 2008; Miller et al., 2009). This suppression of an anti-viral state facilitates slow replication (occurring 5-10 days post-inoculation), establishment of localized PRRSV infection, and failure to activate other innate immune cells such as NK cells, which is in stark contrast to other swine respiratory viruses (van Reeth et al., 1999; Murtaugh et al., 2002). Consequently, the average length of viremia induced by PRRSV infection, which can last up to 21 days or longer, also demonstrates this elongated period of active infection (Albina et al., 1998). This infectious stage can be clinically inapparent in susceptible pigs, making the risk of infecting other naïve animals within production systems very high (Zimmerman, 2006).

Additionally, plasmacytoid dendritic cells (**pDC**), a rare subtype of dendritic cells which are important for the promotion and activation of CTL populations, appear to be negatively impacted by PRRSV. Even though pDC are not permissive to PRRSV infection, it appears that the virus is able to alter pDC function through a negative signal to the cell surface, preventing

co-stimulation of CTL at the time of antigen presentation (Calzada-Nova et al., 2010). Similar to its effects on PAM, reduced type-I interferon signaling and activation of pDCs reduce this population of APC ability to induce naïve T-cells differentiation into interferon-γ (**IFN-γ**) (a potent anti-viral type II interferon) secreting cells and CTL (Loving et al., 2015).

The Porcine Immune Response to PRRSV – The Adaptive Response

Following a delayed and reduced innate immune response, the adaptive immune system responds to PRRSV in two ways: cell-mediated viral clearance or humoral-mediated viral clearance. Regarding cell-mediated responses, T-cells play a critical role in anti-PRRSV immunity through their role in development and regulation of antigen-specific immunity: activation of B-cells, influence on cytokine secretions, cytotoxic effector functions, and regulation of these responses to control inflammation. T-cell populations important for response to PRRSV are cytotoxic T-cells (**CTL**) (CD8+), helper T-cells (CD4+), and memory T-cells (CD4+/CD8+). In general, initial PRRSV infection typically causes a somewhat delayed, transient reduction in helper T-cell populations and a longer increase in CTL populations (Shimizu et al., 1996; Nielsen and Botner, 1997; Albina et al., 1998). This trend is also observed for pigs infected in utero (Feng et al., 2002; Nielsen et al., 2003). Peripheral PRRSV-specific T-cells appear in circulation approximately 2-4 weeks following infection, but levels are variable across time and between individual animals and there does not appear to be any correlation between PRRSV-specific T-cell population sizes and viral load of lymphoid tissues (Xiao et al., 2004). During PRRSV-specific lymphocyte proliferation, helper T-cells appear to predominate, demonstrating type I cytokine expression phenotypes (i.e., secrete IFN-γ and IL-2) which is characteristic of a cell-mediated immune (**CMI**) response induced by intracellular pathogens (López Fuertes et al., 1999). Regardless of age at time of infection, amount of PRRSV-specific

IFN-γ secreting cells are low, which would be expected knowing limitations of the innate immune response to PRRSV (Klinge et al., 2009). Interestingly, cytotoxic effector function of CTL is not strongly linked to control of primary PRRSV infection, with increased CTL activity not observed until much later during infection (Costers et al., 2009). These findings suggest that CTL may be involved in the clearance of PRRSV-infected tissues, though evidence supporting this is inconsistent. Taken together, the inability for complete immunity to PRRSV may be due partly to inadequate T-cell responses, however due to poor representative *in vitro* models for PRRSV infection, it is hard to fully understand the functionality of helper T-cells and CTL under PRRSV-challenge (Loving et al., 2015).

Regarding the humoral response, PRRSV initiates a fairly robust anti-PRRSV antibody response, with detection possible 7-9 days post-infection. There is little evidence that this early antibody production is protective against PRRSV infection and some groups even found evidence of antibody-dependent enhancement (**ADE**) of PRRSV infectivity by anti-PRRSV antibodies (Yoon et al., 1996), though the degree of that contribution to overall pathology is inconsistent (Murtaugh and Genzow, 2011). Evidence of *de novo* synthesis and protection by neutralizing antibodies (**NAb**) against PRRSV has been relatively inconsistent and inconclusive across the literature, with the most promising effects being lowered levels of viremia (Loving et al., 2015). However, serum transfer experiments have been very successful in preventing PRRSV infection of pregnant females and piglets by action of NAb alone. Transfer of NAb in these experiments provided sterilizing immunity for both dams and piglets (Osorio et al., 2002). Separate passive transfer experiments demonstrated that NAb administration and subsequent protection against PRRSV infection was dose dependent, with concentrations under what would achieve antibody titers of 1:8 not providing sterilizing immunity and allowing viral transmission

to naïve pen-mates (Lopez et al., 2007). These findings suggest that NAb may provide effective protection against PRRSV infection, but are likely not synthesized rapidly enough *de novo* to promote clearance. This has made NAb a target for the production of PRRSV vaccines, but their success is highly limited to homologous conditions between the challenge strain of PRRSV and NAb specificity for that strain (Loving et al., 2015).

Keeping the limitations of the PRRSV immune response in mind, the most protective component of the response is adaptive cell-mediated immunity. While significantly delayed due to suppression of type I interferons released by the innate immune response, CMI likely facilitates a majority of viral clearance following infection. An antibody response to PRRSV is evident, with detectable levels present relatively quickly following natural infection, but their efficacy in promoting clearance is inconsistent and has been found to actually increase the period of infectivity for certain PRRSV strains. The major gaps in our knowledge about the immune response to PRRSV are how to circumvent or reduce the inhibition of the initial innate immune response, the specific functionality of helper T-cell and CTL populations following PRRSV-challenge, and how to generate cross-reactivity of NAb against PRRSV strains to reduce limitations currently facing their application as vaccines due to requirement of strain homogeneity. It is also these gaps in knowledge that motivates the investigation of alternative strategies to manage or modulate the immune response to PRRSV, such as nutritional interventions. Among alternatives researched, soy isoflavones have been shown to beneficially alter the immune response to PRRSV in live animal models.

Soy Isoflavones and PRRSV[4]

Due to severity and pervasiveness of PRRSV in the swine industry, there have been several studies using PRRSV as the disease challenge model to assess the effects of feeding soy isoflavones on response to disease and performance. Prior to investigation of isoflavones as an immunomodulatory supplement against PRRSV, an experiment was performed to establish the influence of systemic PRRSV infection in weanling pigs on growth performance and immune responses. When inoculated at 29 d of age, weaned pigs naïve to PRRSV exhibited rapid increases in serum viral concentration and serum interferon (**IFN**) concentrations by 4 d post-inoculation (**DPI**). Both average daily gain (**ADG**) and average daily feed intake (**ADFI**) declined for the first 8 DPI by 47.5% and 22.4%, respectively. These authors went on to demonstrate a negative relationship between serum viral concentration and growth performance measures, indicating that as serum virus concentrations increased, pig performance decreased. The implications of these early findings suggested that immunomodulators with potential to decrease systemic viral concentrations could improve weaned pig performance under disease challenge conditions (Greiner et al., 2000).

After establishing the growth and immune responses to PRRSV infection in weanling pigs, further research sought to investigate whether supplementation with soy isoflavones genistein and daidzein could improve growth performance in the face of an active PRRSV infection. Pigs received purified dietary genistein at 0 to 800 mg/kg and were inoculated with live PRRSV at 29 d of age. Graded dietary intake of genistein caused a linear reduction in serum virus concentrations and a quadratic reduction is serum IFN concentrations, thereby indicating faster viral clearance. Additionally, dietary genistein ingestion improved ADG and ADFI, though

[4]Sections adapted from Smith, B. N. and R. N. Dilger. 2018. Immunomodulatory potential of dietary soybean-derived isoflavones and saponins in pigs. J. Anim. Sci. 96:1288-1304. doi: 10.1093/jas/sky036.

the magnitude of this response was greatest during the early post-inoculation period (4 to 12 DPI) and only when pigs were fed 400 to 800 mg/kg genistein. These improvements in ADG were logarithmically related to serum virus concentration, with each log reduction of serum viral concentration resulting in an improvement of 0.034 kg in pigs averaging 5.3 kg BW and 0.004 kg in pigs averaging 11 kg BW. Two mechanisms of action were suggested, including effects on function and intracellular signaling pathways of immune cells and interruptions in viral replication or attachment (Greiner et al., 2001a). Under the same experimental conditions, dietary daidzein fed at 0 to 800 mg/kg alone did not result in changes in serum virus concentrations. These authors also reported that 200 to 800 mg/kg of dietary daidzein resulted in increased serum IFN concentrations during early periods of high serum virus concentrations (0 to 4 DPI), which is opposite from what was observed for genistein. Admittedly, 200 or 400 mg/kg of dietary daidzein did improve ADG, ADFI, and feed efficiency during periods of high serum virus concentrations (4 to 16 DPI), though these effects were lost as serum virus concentrations decreased (Greiner et al., 2001b). It is important to note that graded ingestion of genistein and daidzein during the pre-inoculation period resulted in linear decreases in ADG, suggesting that supplemental soy isoflavones may not benefit the performance of non-disease-challenged animals. However, the lack of a genistein supplementation and day interaction indicates that feeding genistein prior to inoculation may have aided in the pigs' ability to resist viral infection initially or reduce viral infectivity, as suggested above. That could imply that while supplemental isoflavones do not appreciably benefit growth performance, they may help protect against prolonged performance reduction if that same population were to be exposed to a novel pathogen.

After evaluating the effects of supplementing individual soy isoflavones in disease-challenged pigs, a natural next step was to investigate potential effects of natural soy isoflavone-containing feedstuffs. One study examined the effect of dietary SBM concentrations, a product with moderate-to-high levels of naturally occurring isoflavones, on the growth and immune responses of PRRSV-infected weanling pigs. Following a similar experimental design, weanling pigs receiving either a low SBM diet (**LSBM**, 17.5% SBM, 22.8% CP, 700 mg/kg total ISF) or high SBM diet (**HSBM**, 29.0% SBM, 26.7% CP, 1,246 mg/kg total ISF) were inoculated with active PRRSV. Post-inoculation, PRRSV-infected pigs receiving HSBM diets did not experience a decrease in ADG compared with a non-infected control group, whereas infected pigs receiving LSBM diets did. There were no effects of SBM inclusion on serum viral concentrations at 3 or 7 DPI, but the HSBM-fed pigs did exhibit higher cycle threshold values (i.e., reduced viral concentrations) at 14 DPI compared with LSBM-fed pigs. Most inflammatory parameters were not affected by SBM concentration under PRRSV-challenge, though HSBM-fed pigs did have higher hematocrit concentrations than LSBM-fed pigs at 14 DPI, possibly indicating lessened stress on red blood cell reserves throughout the infection process (Rochell et al., 2015). Although higher concentrations of SBM in the diet improved growth in PRRSV-infected pigs, this study's outcomes were limited by not being able to differentiate beneficial effects from biologically active compounds like isoflavones and increased crude protein or AA availability.

In order to differentiate these effects, further research investigating soy isoflavones was performed by our laboratory and those findings are discussed in following data chapters. While our research does suggest that improved performance under PRRSV-challenge may be in part due to soy isoflavones, we recognize that isoflavones are dietary compounds partially subject to metabolism by organisms other than the animal to which they are fed: the gastrointestinal

microbiome. For that reason, we have to take into account the potential role the gastrointestinal microbiome plays in the response to systemic disease and how soy isoflavones may influence that response.

The Microbiome, PRRSV, and Soy Isoflavones

Factors That Influence Swine Gastrointestinal Microbiome Development

With increasing evidence showing that nutritional interventions can influence whole-body health, the role of the gastrointestinal microbiome has garnered great attention. Not only is the gastrointestinal microbiome responsible for the metabolism of a multitude of compounds that are non-digestible/metabolizable by mammalian enzymes, it is a dynamic population responsive to changes in dietary composition and is directly able to interact with the host (Quigley et al., 2013; Frese et al., 2015). One important interaction the microbiome has with its host involves the development and regulation of the immune system, which has long-term health implications for growing pigs. Considering that soy isoflavones fall into the category of dietary components only partially metabolized by mammalian enzymes (Cassidy et al., 2006), understanding their impact on the gastrointestinal microbiome and how the microbiome changes the metabolism and bioavailability of isoflavone metabolites in both healthy and diseased pigs is an area of interest, especially when we consider their immunomodulatory potential.

Knowledge of the development of the gastrointestinal microbiome in growing pigs is essential to understanding the influence diet can have on its composition. As the weaned pig transitions from a diet comprised solely of sow's milk to a more complex diet comprised of mainly plant-based components, populations comprising their intestinal microbiome also undergo transitions. According to metagenomic sequencing data, microbial community diversity (i.e., alpha diversity, a measure of the number of different species detected within a microbial ecosystem) gradually increases starting at birth, but that diversity quickly shifts based on diet

composition. During nursing (i.e., days 1-21 of life), microbial communities are fairly stable with the most abundant families being *Bacteroidaceae* (aka. *Bacteroides*), *Clostridiaceae, Lachnospiraceae, Lactobacillaceae,* and *Enterobacteriaceae*. From weaning until 7 weeks of age, populations of *Bacteroidaceae* and *Enterobacteriaceae* decline and populations of *Lactobacillaceae, Ruminococcaceae, Veillonellaceae,* and *Prevotellaceae* increase (Frese et al., 2015), though there is some evidence that weaning may decrease some *Lactobacillaceae* subspecies (Niederwerder, 2017). Among pigs from approximately 3 weeks of age (i.e., weaning) until slaughter age (~24 weeks), *Firmicutes* and *Bacteroides* phyla accounted for nearly 85% of total 16S rRNA sequences across the gastrointestinal tract. Moreover, genera including *Clostridium*, *Blautia*, *Lactobacillus, Prevotella*, *Ruminococcus*, *Roseburia*, and *Subdoligranulum* were present in more than 90% of samples analyzed with *Prevotella* being the most abundant (Holman et al., 2017). While diet maintains the strongest influence over microbial composition, other factors that can influence the microbiota of growing pigs include host genetics, environment, mode of delivery (i.e., vaginal vs. caesarean), microbiome of the sow, antibiotic exposure, pathogenic stressors (particularly enteric pathogens), and handling/processing stressors (Niederwerder, 2017). Of these factors, pathogenic stressors are of particular concern for swine production, especially around the time of weaning when risks of encountering enteric and respiratory pathogens is high.

The Microbiome, the Immune System, and the Gut-Lung Axis

Not only is the gastrointestinal microbiome a dynamic population and responsive to external factors, it itself exerts its own influence on its host. One particularly important relationship between the gastrointestinal microbiome and the host is its direct influence on the immune system's development and function both locally and systemically. These influences

include directly impacting the development of the innate immune system, specifically helping maintain homeostasis and epithelial function at mucosal surfaces, influencing T-cell differentiation, modulating inflammation, and regulating the adaptive immune response (Kau et al., 2011; Hooper et al., 2012; Hauptmann and Schaible, 2016; Niederwerder, 2017). These effects are driven by microbial population composition and in part by their unique metabolic activity through the production of secondary metabolites by fermentation (Collado et al., 2018).

This role is further emphasized by gnotobiotic animal models; in "germ-free" animals (i.e., those reared in an environment devoid of microbial organisms) where a microbiome is unestablished, tissues and organ systems associated with the immune system such as lymph nodes are less developed and these animals typically exhibit more severe clinical disease lesions in the presence of a pathogen challenge (Niederwerder, 2017). Even in animal models where a host microbiome has been established but is greatly depleted by the use of antibiotics we can observe immune system dysfunction. For example, in a microbiome-depleted mice model investigating the role of the microbiota in the host response to pneumococcal pneumonia, researchers found that microbiome-depleted mice had accelerated mortality rates, increased local lung and circulating bacterial concentrations, and increased pro-inflammatory IL-1β and IL-6 serum cytokine concentrations compared to controls (Schuijt et al., 2016). In general, studies of this nature identified associations between increased microbiome diversity and improved clinical outcomes. Other studies have gone a step further and identified specific bacterial types that were found to be associated with immune responses to pathogen challenge. For example, under challenge with respiratory pathogens, increased relative abundance of lactic acid producing bacteria such as *Lactobacillus* species has been showed to increase NK cell activity, reduce pro-inflammatory cytokine production, enhance antiviral responses, upregulate cell-mediated

cytotoxicity, and increase mucosal antibody production. Increased relative abundance of *Bifidobacterium longum* has been shown to reduce clinical signs, decrease weight loss, lower viral replication, and reduce lung pathology in *in vivo* influenza models (Niederwerder, 2017). While it seems reasonable that the microbiome and its constituents may influence the immune system locally, such as innate immune function including epithelial integrity and antigen recognition by innate immune cells at the level of the gut (Hooper et al., 2012; Niederwerder, 2017), its ability to affect immune function systemically, as described by the studies referenced above, is in some ways unexpected. This ability has been the focus of numerous research groups, particularly regarding the connection between the gastrointestinal microbiome and respiratory illness.

Through continued research of this relationship, a well-documented phenomena known as the "gut-lung axis" has been established, which describes a bi-directional communication between microbial populations of the gastrointestinal and respiratory tracts. Within this context, the gastrointestinal and respiratory tracts have been shown to share a "common mucosal response", which involves effects of the gastrointestinal microbiome on mucosal immunity driving immune responses at distal mucosal sites such as the lungs (Mcghee and Fujihashi, 2012; Hauptmann and Schaible, 2016; Date et al., 2017). Perhaps most interestingly, several dysbioses of the gastrointestinal microbiome have been associated with lung disorders and respiratory infections in humans, including viral pathogens (Collado et al., 2018). In respiratory viral pathogen models, microbiome composition and diversity has been shown to be directly associated with clinical outcomes including diseased progression, airway inflammation, immune response, and morbidity (Ober et al., 2017). Shifting focus back to swine production and health, the influence of the microbiome on immune system function and the relationship between the

microbiome and the respiratory tract is an area of new discussion for how nutritional interventions, like soy isoflavones, may modulate the immune response and improve productivity in pigs facing a respiratory pathogen challenge.

Microbiome Response to PRRSV

Due to increased interest in this area of research, there are recent studies evaluating the relationship between gastrointestinal microbiome composition and swine respiratory disease. Specifically focusing on disease models including a PRRSV-challenge component, there are notable differences in pig performance and clinical outcomes that correspond with differences in microbiome composition. In a PRRSV and porcine circovirus type 2 (**PCV2**) co-infection model in weanling pigs, researchers found that pigs with the best clinical outcomes (i.e., highest ADG from 0 to 63 DPI and lack of overt clinical disease) had greater fecal microbial diversity than pigs with the worst clinical outcomes (i.e., lowest ADG from 0 to 63 DPI and at least 10 days of moderate to severe clinical disease). In the worst clinical outcomes group, pigs experienced delayed peak PRRSV-infection with prolonged and increased viremia, higher peak PCV2 viremia levels (non-significant), and increased serum concentrations of *Bacillus cereus*, suggesting these animals experienced a more severe systemic infection and compromised mucosal integrity. Regarding microbiome differences observed, in addition to differences in overall measures of microbiome diversity, pigs in the worst clinical outcomes group notably had no *Escherichia coli* (***E. coli***) detected in their fecal samples. Though swine are susceptible to a number of pathogenic strains of *E. coli*, in respiratory and gastrointestinal pathogen challenge models commensal *E. coli* presence has been associated with greater feed efficiency, greater weight gain, and reduced cachexia. Additionally, bacterial populations of the Proteobacteria phylum were only found in fecal samples from pigs in the best clinical outcome group, which

has been associated with improved clinical outcomes in other pathogen challenge models in swine (Niederwerder et al., 2016).

Continuation of this research resulted in similar findings when researchers investigated what early microbiome properties may influence growth performance following co-infection in PRRSV/PCV2 co-infected pigs. They found that following subclinical infection with these two respiratory pathogens there was a clear divergence of between high and low growth rate groups starting at approximately 21 DPI. Pigs that fell within the high growth rate group had reduced PRRSV and PCV2 replication rates and decreased lung lesion severity compared to the low growth rate group, which was in accordance with previous findings. Also similar to previous findings, pigs that fell within the high growth rate group had greater fecal microbial diversity at the time of infection as well as increased species diversity in *Ruminococcaceae* and *Streptococcaceae* families, both of which have been previously associated with improved growth performance in swine (Ober et al., 2017). These data suggested that microbiome composition not only influences clinical outcomes during an active respiratory infection, but may confer these benefits as early as first exposure to these respiratory pathogens.

Microbiome Response to Soy Isoflavones

In that context, if increased microbial diversity is associated with better clinical outcomes in swine, knowing if nutritional interventions alter microbial composition of the animals to which they are fed is also of interest. Regarding soy isoflavones, most research evaluating their effect on the gastrointestinal microbiome has been conducted in humans. That said, dietary soy isoflavones have been shown to influence the relative proportions of several bacterial populations across different studies. In post-menopausal women, isoflavone consumption increased the relative proportions of a number of bacterial populations including

Bifidobacterium, *Lactobacillus,* and *Eubacterium*, though significant inter-individual variation occurs and effects do appear to dose-dependent (Nakatsu et al., 2014; Huang et al., 2016; Kolátorová et al., 2018). In a human breast cancer mice model, genistein alone increased butyrate-producing bacterial families, which is associated improved colon health, suppressed expression of pro-inflammatory/pro-oxidative cell signaling pathways, and increased expression of anti-inflammatory cell signaling pathways (Paul et al., 2017), which suggests that isoflavones may beneficially alter the microbiome in the context of certain disease processes. *In vitro* studies evaluating the effects of isoflavones and their associated metabolites on the growth of common gastrointestinal bacterial strains suggests that isoflavones may exhibit positive growth pressure on bacterial strains that are able to metabolize them or utilize them as an energy source (Vázquez et al., 2017). It is unclear how these effects would translate to swine, which are exposed to dietary isoflavones throughout their productive life, particularly when considering a majority of these findings are in mature females alone, which excludes interactions with growth and development and sex that may influence the ability of isoflavones to influence microbiome composition (Kolátorová et al., 2018).

It is also important to note that a majority of these human studies focus on the potential differences of individuals to produce equol, a secondary metabolite of daidzein that is highly bioactive, more so than any other isoflavone or isoflavone metabolite. Equol is an isoflavandiol, nonsteroidal estrogen-like compound that maintains high biological activity. In *in vitro* studies, equol out competed both genistein and daidzein for growth inhibition of several fungal and common bacterial types, demonstrating the greatest antioxidant capacity among all isoflavones and their metabolites (Chang et al., 1995; Setchell et al., 2002). As a metabolite, equol appears to be relatively stable and tolerant of peripheral circulation in the body in its biologically active

form better than parent isoflavones themselves. However, the ability to synthesize equol is highly variable between species and individuals, largely owing to differences in composition of gastrointestinal microbial populations. In humans, only 30-50% of test populations have been found to be “equol-producers” (Setchell et al., 2002). Within swine populations, it appears that some have microbiomes that support equol production (Yu et al., 2008), but among U.S. breeds it seems more variable. A study evaluating equol production and contents in feces and urine from large white sows showed results similar to what had been demonstrated in humans with high inter-individual variability. Like humans, individual sows that had larger detectable levels of equol in their feces or urine had similar microbial population phenotypes to each other (Zheng et al., 2017). Moving forward, it is pertinent to understand how the microbiome populations in swine are influenced by isoflavones and if they are, do those differences correspond with increased microbial diversity or enhanced performance when facing a respiratory pathogen challenge.

Table

Table 2.1. Isoflavone concentrations (mg/kg of product) of common soybean-derived products[1]

Item (mg/kg)	Soybean meal	Soybean protein concentrate	Soybean protein isolate
Genistein	1,147	52.6	573
Daidzein	808	57.8	308
Glycitein	161	15.7	85.4
Total Isoflavones	2,096	115	911

[1]Values obtained from the USDA Agricultural Research Service Nutrient Data Laboratory (USDA-ARS, 2016)

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CHAPTER 3: EFFECTS OF DIETARY SOY ISOFLAVONES AND SOY PROTEIN SOURCE ON RESPONSE OF WEANLING PIGS TO PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRAL INFECTION[5]

Abstract

Porcine reproductive and respiratory syndrome virus (**PRRSV**) is the most prevalent disease of swine globally. Infection of weanling pigs with PRRSV leads to a complex immune response resulting in significant disease and decreased growth performance. Previous experimental evidence suggests that increasing concentrations of soybean meal in the diet of young pigs confer benefits in terms of growth performance and immune parameters. The objective of this experiment was to identify potential modes of action for this benefit, specifically the ability for soy-derived isoflavones (**ISF**) to confer immunological benefits to young pigs infected with PRRSV. Four dietary treatments differing in soy protein source (soy protein concentrate vs. enzyme-treated soybean meal) and ISF supplementation (none vs. 1,500 mg total ISF/kg) were fed; the control diet (**CON**) contained soy protein concentrate and no supplemental ISF. Weanling pigs (60 barrows, 21 d of age, 5.71 ± 0.44 kg) from a naturally *Mycoplasma hyopneumoniae* (**Mh**)–infected source herd were individually housed in disease containment chambers and provided ad libitum access to experimental diets for 7 d before receiving either a sham inoculation or a 9.28×10^3 50% tissue culture infective dose of PRRSV at 28 d of age (0 d post-inoculation, **DPI**). A total of 5 experimental treatments included an uninfected group receiving the CON diet, plus four infected groups each receiving a different dietary treatment. Growth performance and rectal temperatures were recorded throughout the study, and blood was

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collected for quantification of serum PRRSV load, presence of anti-PRRSV antibodies, differential complete blood counts, cytokine concentrations, and T-cell immunophenotyping. Data were analyzed as a 2-way or 3-way ANOVA for all treatments including PRRSV-infected pigs, in addition to a single degree of freedom contrast to compare uninfected and infected pigs receiving the CON diet. PRRSV-infection reduced growth rate and efficiency compared with non-infected controls with minimal influences by ISF. Supplemental ISF reduced PRRSV-induced band neutrophilia and improved cytotoxic-to-helper T-cell ratios. These results suggest that ISF contribute to activation of adaptive immune system pathways and could benefit recovery from and clearance of PRRSV infections.

Introduction

Porcine reproductive and respiratory syndrome virus (**PRRSV**) continues to be one of the leading pathogens causing high economic impact on modern swine production. Previous studies estimated that production losses within breeding and growing swine herds to be more than US$650 million per year, costing producers US$3 to US$11 per pig infected (Holtkamp et al., 2013; Schweer et al., 2017). Controlling spread and eradication of PRRSV has remained a challenge due to ease of transmission between animals, heterogenic properties of the virus itself, and its ability to persist within an animal host and environment. Although vaccination protocols are available, their efficacies vary and often do not result in complete sterilizing immunity (Loving et al., 2015). For those reasons, nutritional intervention of or management strategies is of high interest for common diseases affecting livestock due to relatively low cost and ease of implementation. It has been demonstrated that increasing the level of soybean meal in the diet fed to pigs under immune stress reduces adverse effects of illness and may improve growth performance (Boyd et al., 2010; Rochell et al., 2015). However, it remains to be seen whether

improved growth performance was due to differences in dietary amino acids or one or more other dietary components, though there appears to be evidence that differences are likely not due to differences in nutrient digestibility (Schweer et al., 2018). Isoflavones (**ISF**) are flavonoid compounds that are enriched in soybeans and possess anti-inflammatory and anti-oxidative properties (Smith and Dilger, 2018). With that in mind, the objective of this study was to determine whether soy-derived ISF, fed at dietary concentrations comparable to a commercial diet fed to weanling pigs, would demonstrate protective effects against a PRRSV-challenge. It was also of interest to determine whether soy protein source influenced the efficacy of dietary ISF modulating a disease challenge in weaning age pigs.

Materials and Methods

The protocol for this experiment was approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of the University of Illinois at Urbana – Champaign.

Animal Husbandry and Experimental Design

Sixty weanling barrows (5.71 ± 0.44 kg initial body weight) were obtained from a PRRSV-negative, non-PRRS vaccinated commercial herd (1050 Cambro genetics; Carthage Veterinary Service, Ltd.) and housed in disease containment chambers in the Edward R. Madigan Laboratory at the University of Illinois (Urbana, IL) for 21 d (−7 to 14 d post-inoculation, **DPI**) immediately after weaning at approximately 21 d of age. A single corridor of containment chambers was used, with access to 8 chambers each housing 4 individual pigs, and the total number of pigs was split into 2 separate cohorts (n = 28 and n = 32 in cohorts 1 and 2, respectively) that were conducted in successive months. Each chamber (3.34 m^2 total floor

space) was divided into 4 individual pens (0.84 m^2 per pen) and each was equipped with 1 nipple waterer and 1 feeder.

Experimental diets were provided beginning at the time of allotment. Pigs were weighed upon arrival for allotment into 5 experimental treatment groups. Pigs were assigned to dietary treatments and allotted to containment chambers (blocks) based on body weight and litter so that weight distributions were similar within a chamber across all treatment groups. Litter of origin (14 litters total across the 2 cohorts) was taken into account, and pigs from each litter were stratified across treatment groups as evenly as possible. This allotment resulted in 12 pigs for each treatment group, with each chamber having 1 replicate pig per dietary treatment with the exception of the uninfected group (3 blocks total). One intramuscular injection of enrofloxacin (7.5 mg/kg BW; Baytril 100; Bayer, Shawnee Mission, KS) was administered on the day pigs arrived as a prophylactic measure against bacterial infections during transition to the new rearing environment. Pigs were provided their assigned experimental diet and allowed to adjust to housing conditions for 7 d prior to initiating inoculation procedures. Lights were maintained on a 12-h light cycle throughout the study, with light provided from 0600 to 1800 h in a thermostatically controlled environment with containment chamber temperatures set at 28–29 °C throughout the study.

As stated, 5 experimental treatments were used in this study, with 4 different diets and 2 states of infection. A 2 × 2 + 1 factorial arrangement of dietary soy protein sources (soy protein concentrate [**SPC**], Arcon AF, ADM, Decatur, IL vs. enzyme-treated soybean meal [**ETSBM**], HP300; Hamlet Protein, Findlay, OH) and supplemental ISF (none vs. Novasoy400; ADM, Decatur, IL) constituted the total of dietary treatments (**Table 3.1**). Isoflavones were added to the test diets at levels that would be typical for a commercially relevant corn-SBM diet fed to pigs

with approximately 20% SBM inclusion. The control diet contained SPC as a protein source with no addition of soy ISF, and this diet was fed to both sham-inoculated and PRRSV-infected groups. Experimental diets were isocaloric and, with the exceptions of corn and protein source, identical in ingredient composition. Isoflavone and saponin concentrations of ingredients and experimental diets were quantified via HPLC at the USDA–ARS National Center for Agricultural Utilization Research (Peoria, IL) according to procedures of Berhow et al. (2006). Crude protein was determined by measuring nitrogen using a Leco analyzer (TruMac N, Leco Corp., St. Joseph, MI) standardized with EDTA and amino acid concentrations were determined at the University of Missouri Agricultural Experiment Station (Columbia, MO; **Table 3.2**) according to AOAC (2002) official methods [920.39 and 982.30 E(a, b, c), for crude protein and amino acid concentrations, respectively]. Gross energy of the experimental diets was determined using an adiabatic bomb calorimeter (Parr Instruments, Moline, IL), and DM (method 934.01, AOAC International, 2002) and OM were performed by determining percent ash (method 942.05, AOAC International, 2002) and subtracting from 100. Diets were analyzed for total dietary fiber according to Prosky et al. (1994), but no separation of soluble and insoluble fractions was made. Diets were formulated on a standardized ileal digestible (**SID**) amino acid basis with identical concentrations across all diets (**Table 3.3**). All diets met or exceeded NRC (2012) nutrient requirements for weanling pigs and analyzed dietary concentrations are presented in **Table 3.4**.

Following a 1-wk adaptation period to experimental diets, blood was collected from each pig to establish 0 DPI baseline measurements and to ensure that all pigs were PRRSV-negative at study initiation. Immediately following blood collection, pigs were administered via intranasal inoculation either 2 mL of a 2% fetal bovine serum + phosphate-buffered saline solution (sham-

control) or 9.3×10^3 50% tissue culture infective dose (**$TCID_{50}$**) of PRRS virus (strain NADC20, courtesy of Dr. Federico Zuckermann, University of Illinois, Urbana, IL).

Although the model was designed to only utilize PRRSV as the immune challenge, the source herd for piglets used for this study tested positive for *Mycobacterium hyopneumoniae* (**Mh**) after completion of the first cohort and prior to delivery of pigs for the second cohort. We confirmed Mh status in individual pigs by quantitative real-time polymerase chain reaction (**qRT-PCR**) detection of the bacterium in lung tissue only from pigs included in the second cohort, but it is likely that pigs from the first cohort were also harboring the bacterium based on the length of time required for Mh to establish infection and present associated clinical signs (Maes et al. 2018). It should be noted that all but 2 pigs from the second cohort, including those not infected with PRRSV, tested positive for Mh specifically, though the 2 Mh-negative pigs did test positive for general Mycoplasma by qRT-PCR.

Growth Performance, Rectal Temperatures, and Blood Collection

Individual pig and feeder weights were recorded weekly throughout the study to allow for calculation of average daily gain (**ADG**), average daily feed intake (**ADFI**), and feed efficiency (gain to feed, **G:F**). Growth performance data are reported in reference to the inoculation schedule (−7 to 14 DPI). Rectal temperatures of all pigs were measured on 0, 3, 6, 8, 12, and 14 DPI using an over-the-counter, consumer-grade digital thermometer in the morning prior to any blood sample collection.

Blood (up to 8 mL total) was collected from the jugular vein of each pig into evacuated tubes (BD, Franklin Lakes, NJ) at 0, 3, 6, 12, and 14 DPI using a 21-gauge needle. Blood samples from 0 DPI were collected immediately before inoculation. Three milliliters of blood were collected into tubes containing EDTA as an anti-coagulant (i.e., whole blood), placed on

ice, and submitted to the University of Illinois Veterinary Clinical Pathology Laboratory for analysis as described in the following section. Three milliliters of blood were collected into serum tubes, allowed to clot at room temperature, and centrifuged at 1,300 × *g* for 15 min at 20°C. Serum was removed from centrifuged samples and stored in 0.5 mL aliquots at -80°C pending subsequent analyses. On 12 DPI, a total of 8 mL of whole blood were collected from each animal, placed on ice, and used for isolation of peripheral blood mononuclear cells (**PBMC**) for immunophenotyping procedures as described below.

Blood and Sera Measurements

At 0, 3, 6, and 14 DPI, a multi-parameter, automated hematology analyzer (CELL-DYN 3700, Abbott Laboratories, Abbott Park, IL) was used to determine the total and differential cell counts of whole blood by the University of Illinois Veterinary Clinical Pathology Laboratory. Serum from the same experimental time points was submitted to the University of Illinois Veterinary Diagnostic Laboratory to be analyzed for PRRSV load at 0, 3, 6, and 14 DPI by qRT-PCR using Z-PRRSV Multiplex reagents (Tetracore, Rockville, MD) and identical extraction conditions and a single quantity of starting RNA material for all experimental samples. Both positive and "no-template" controls were run along the experimental samples. The assay used a single-tubed method based on fluorogenic probe hydrolysis (TaqMan, Applied Biosystems, Foster City, CA). Viral load was expressed as cycle threshold (**Ct**) values, where a higher Ct value represents a lower amount of PRRSV mRNA. Additionally, a PRRSV antibody screen by ELISA was performed on qRT-PCR-positive samples collected on 6 and 14 DPI for detection of anti-PRRSV antibodies. The results for this assay are expressed as a sample to positive ratio (**S/P ratio**) that represents the following relationship: [*sample mean × (mean of optical absorbance) − negative control mean]/(positive control mean − negative control mean)*]. An S/P ratio greater

than 0.4 is considered a positive result and the larger the number, the more anti-PRRSV antibodies that are present. Finally, serum concentrations of cytokines tumor necrosis factor-α (**TNF-$\boldsymbol{\alpha}$**), interferon-γ (**IFN-$\boldsymbol{\gamma}$**), interleukin-1β (**IL-1$\boldsymbol{\beta}$**), interleukin-10 (**IL-10**), interleukin-8 (**IL-8**), interferon-α (**IFN-$\boldsymbol{\alpha}$**), and interleukin-4 (**IL-4**) were also measured at 0, 3, 6, and 14 DPI with the Swine Cytokine Magnetic 7-Plex Panel according to manufacturer instructions (Novex by Life Technologies, Frederick, MD).

T-Cell Immunophenotyping

Whole blood collected on 12 DPI was used for isolation of PBMC for a T-cell immunophenotyping procedure using flow cytometry. In brief, PBMC were isolated by placing individual whole blood samples over a density gradient (SepMate 15 [IVD] and Lymphoprep, StemCell Technologies, Cambridge, MA) and centrifuging at 1,200 × *g* for 25 min at 20°C. Isolated PBMC were then washed with phosphate buffer saline containing 2% fetal bovine serum before being counted using the Moxi Z Mini Automated Cell Counter (ORFLO Technologies, Ketchum, ID). Individual samples were prepared with 1.0×10^6 cells and treated with a protein transport inhibitor (BD Pharmingen, San Jose, CA) 4 hours at 37°C. Following this treatment, cells were incubated with a 2% porcine serum blocking solution for 15 minutes at 4°C. Cells were then labeled with external fluorescent antibodies against cell surface markers CD3 (PE-Cy7 Mouse Anti-Pig CD3ε, BD Pharmingen, San Jose, CA), CD4 (Alexa Fluor 647 Mouse Anti-Pig CD4a, BD Pharmingen, San Jose, CA), and CD8 (PE Mouse Anti-Pig CD8b, BD Pharmingen, San Jose, CA). Following application of external antibodies for 45 minutes at 4°C, cells were permeabilized using the BD Perm/Wash Buffer (BD Biosciences, San Jose, CA) and fixed using 4% paraformaldehyde solution for 20 minutes at 4°C. The following day, cells were labeled with an intracellular fluorescent antibody against IFN-γ (PerCP5.5 Mouse Anti-Pig IFN-γ, BD

Pharmingen, San Jose, CA). Prepared, labeled cells were evaluated at the University of Illinois Flow Cytometry Facility using a BD LSR II Flow Cytometry Analyzer (BD Biosciences, San Jose, CA) and flow cytometer outputs were analyzed using FCS Express 5 Plus (De Novo Software, Glendale, CA).

To summarize our analysis and gating procedures, recorded cellular events were plotted by density using forward and side scatter areas on the y- and x-axes, respectively, to exclude dead cells and cellular debris and allow for the selection of lymphocyte cells based on size. A single gate was applied to the lymphocyte population, the content of which was then plotted by density using forward scatter width and side scatter on the y- and x-axes, respectively, to allow for the gating and analysis of single cell events within our lymphocyte population only. Utilizing single cellular events within our lymphocyte population, single-stain control samples were used to set our detection thresholds for each individual fluorochrome and creation of a CD3+ gate. Gated on CD3+ lymphocytes, a final density plot using our CD8+ and CD4+ fluorochrome channels on the y- and x-axes, respectively, was created and our individual fluorochrome detection levels were used to apply quadrants to the density plot. Statistics from each quadrant were then used for our individual effector T-cell proportions (e.g., Helper T-cells, CD3+/CD4+).

Statistical Analyses

Statistical analysis was dependent on whether outcomes were measured at a single time point or at multiple time points for the same subject. Apart from growth performance outcomes, individual pig was considered the experimental unit, with 12 replicate pens or pigs for each of the 5 experimental treatments. A total of 4 different diets were fed to PRRSV-infected pigs, whereas a single group of uninfected pigs received only the control diet. Thus, the 2- or 3-way analyses of variance described below involve effects between diets and within PRRSV-infected

pigs, whereas a separate analysis was used to compare uninfected and infected groups receiving the control diet. In all cases, interaction means are presented in data tables, whereas significant main effect means are presented in data figures.

For all single time-point outcomes, a 2-way ANOVA was conducted using the MIXED procedure of SAS 9.3 (SAS Institute, Inc., Cary, NC) with factors including the dietary soy protein source (SPC vs. ETSBM) and addition of supplemental soy ISF (no vs. yes). Comparison of uninfected and infected groups each fed the control diet was made using a single degree of freedom contrast between these experimental treatments.

With regard to repeated measures outcomes, a 3-way ANOVA was conducted for all outcomes involving samples collected from the same subject at multiple time points with factors including the dietary soy protein source (SPC vs. ETSBM), addition of supplemental soy ISF (no vs. yes), and time (DPI). Repeated measures were sliced by DPI for main effect and interaction means and those P-values are included in data tables. No 2-way or 3-way interactive effects involving DPI were significant, so these effects were removed from the statistical model. Comparison of uninfected and infected groups each fed the control diet was made using a single degree of freedom contrast between these experimental treatments. In all cases, outliers were identified as having an absolute Studentized residual value of 3 or greater and significance was accepted with at $P \leq 0.05$.

Results

Growth Performance

Growth performance results are shown in **Table 3.5**. Within the PRRSV-infected groups, no main or interactive effects were noted during the pre-inoculation period ($P > 0.05$; −7 to 0 DPI). However, significant decreases ($P < 0.01$) in ADG were observed between uninfected and

infected groups fed the control diet throughout the post-inoculation period, thereby signifying a successful PRRSV-infection. During the infective period, there were main effects of ISF supplementation on G:F between 6 and 14 DPI and main effects of soy source on ADG and G:F over the entire 2-wk infection period ($P < 0.05$; 0 to 14 DPI). From 6 to 14 DPI, pigs receiving the ETSBM + ISF diet experienced less efficient growth compared with pigs receiving only ETSBM (173 vs. 484 g/kg, respectively; $P < 0.05$). Over the 2-wk infection period, pigs receiving the ETSBM diet maintained a higher ADG compared with pigs fed the Control diet, regardless of ISF supplementation (219 vs. 137 g/d, respectively; $P < 0.05$). Additionally, pigs receiving ETSBM, regardless of ISF supplementation, had greater feed efficiency compared with pigs receiving the Control + ISF diet over the 2-wk infection period ($P < 0.05$). Overall, PRRSV-infection reduced ADG and G:F during the 2-wk infection period (0 to 14 DPI) resulting in a lower final body weight ($P < 0.0001$) for PRRSV-infected pigs compared with uninfected pigs fed the Control diet.

Rectal Temperatures

Rectal temperature data were collected frequently over the entire infection period (0 to 14 DPI; **Table 3.6**). Infection with PRRSV increased ($P < 0.05$) rectal temperatures of pigs at 0, 3, 6, and 8 DPI, but there was no influence ($P > 0.05$) of dietary soy source or ISF supplementation on this outcome.

Serum Viral Load and Anti-PRRSV Antibody Presence

Results for serum viral load and presence of anti-PRRSV antibody assays can be found in **Table 3.7**. Analysis of serum by qRT-PCR indicated that all pigs were PRRSV-negative at 0 DPI. At 3, 6, and 14 DPI, all pigs in the uninfected group remained free of PRRSV as shown by negative qRT-PCR results, whereas all pigs inoculated with PRRSV tested positive for PRRSV

mRNA. Among the PRRSV-infected groups, a main effect of ISF was observed at 3 DPI with Ct values, the number PCR cycles required to detect presence the viral genome, being lower (i.e., more viral mRNA copies present) in pigs receiving supplemental ISF than those pigs un-supplemented (14.27 vs. 17.19, respectively; $P < 0.05$; **Figure 3.1**). Additionally, there was an interaction main effect observed at 3 DPI with pigs fed the ETSBM + ISF diet having lower Ct values compared with pigs in all other groups ($P < 0.05$).

The presence of anti-PRRSV antibodies was also assessed on qRT-PCR-positive samples collected on 6 and 14 DPI by IDEXX-ELISA (**Table 3.7**). As a reminder, an S/P ratio greater than 0.4 is considered a positive result and the larger the number, the more anti-PRRSV antibodies that are present. Although there were no main or interactive effects observed at 6 DPI, a main effect of soy source was observed on 14 DPI ($P < 0.05$). Pigs receiving the Control diets had greater concentrations of anti-PRRSV antibodies than those receiving the ETSBM diets, regardless of ISF supplementation (1.649 vs. 1.458, respectively; $P < 0.05$). Please note that this assay was for total IgG anti-PRRSV antibody presence and was not specific for neutralizing antibody types.

Red Blood Cell Measurements

Results for red blood cell measurements can be found in **Table 3.8**. Between non-infected and infected pigs, PRRSV-infection resulted in reductions of red blood cell counts (**RBC**, 1×10^6 cells/mL), hemoglobin concentrations (**HGB**, g/dL), hematocrit (**HCT**, %), mean corpsucular volume (**MCV**, fl), and mean corpuscular hemoglobin (**MCH**, pg) at 14 DPI ($P < 0.05$), but no other time points were affected. No main effects of soy source or ISF supplementation were observed for any red blood cell parameters. The only significant interaction effect between soy source and ISF supplementation was for mean corpuscular

hemoglobin concentration (**MCHC**, g/dL) at 14 DPI. PRRSV-infected pigs receiving the ETSBM + ISF diet had lower MCHC compared with all other infected groups ($P < 0.05$).

Leukocyte Measurements

Results for leukocyte measurements are shown in **Table 3.9**. Regarding relative proportions of individual white blood cell (**WBC**) populations, PRRSV-infection resulted in leukopenia at 6 and 14 DPI, neutrophilia at 6 and 14 DPI, greater circulatory concentrations of band cells (i.e., circulating immature neutrophils; **BAND**) at 3 and 6 DPI, and lymphopenia at 3, 6, and 14 DPI when comparing non-infected and infected control pigs ($P < 0.05$). Infected control pigs also exhibited greater monocyte populations than non-infected controls, though this was only observed at 0 DPI ($P < 0.05$). There were main effects of ISF supplementation for band cells. Animals receiving supplemental ISF had lower ($P < 0.05$) concentrations of circulatory band cells, though this effect was only observed at 3 DPI (2.14% vs. 6.32%, respectively; **Figure 3.2**). There were main effects of soy source for total WBC counts and eosinophil concentrations. Animals receiving the ETSBM diet without supplemental ISF had greater ($P < 0.05$) WBC counts than all other treatments at 6 DPI, but this effect was not maintained at any other time point (19.47×10^3 cells/μL vs. 12.32×10^3 cells/μL, respectively; **Figure 3.2**). Additionally, pigs fed the ETSBM diet had lower ($P < 0.05$) circulatory eosinophil concentrations compared with pigs receiving the Control diet at 3 DPI, with pigs receiving the Control + ISF having the greatest (1.17% vs. 3.03%, respectively). There were interaction effects for WBC counts and eosinophils. Within infected groups, pigs receiving the ETSBM + ISF diet experienced the least severe ($P < 0.05$) leukopenia at 6 DPI (i.e., smaller reduction in WBC counts) compared with all other treatments. Additionally, as previously discussed for the main effect of soy source, pigs

receiving the Control + ISF treatment had greater ($P < 0.05$) eosinophil concentrations than both ETSBM treatments, regardless of ISF supplementation.

Serum Cytokine Concentrations

Serum cytokine concentrations, quantified using a porcine-specific multiplexed ELISA, are shown in **Table 3.10**. Due to large numbers of samples with low or absent detection, cytokines IL-1β, IL-8, and IL-4 were omitted from analysis. PRRSV-infected pigs had higher circulating concentrations of IFN-α and TNF-α at 3 and 14 DPI, respectively, compared with uninfected controls. No other cytokines or time points were influenced by infection status. There were main effects for both soy source and ISF supplementation and interaction effects for IFN-α concentration at 3 DPI (**Figure 3.1**). Pigs that received the ETSBM + ISF diet expressed the greatest ($P < 0.05$) concentrations of IFN-α when compared with pigs fed the Control diet, regardless of ISF supplementation. Additionally, there were interaction and main effects for ISF supplementation on IFN-γ at 3 DPI (**Figure 3.1**). Pigs receiving the Control diet without supplemental ISF concentrations at 3 DPI had greater ($P < 0.05$) IFN-γ than all other treatments, though this effect was not observed at any other time point.

T-Cell Immunophenotyping

Results for immunophenotyping analysis of PBMC using flow cytometry can be found in **Table 3.11**. Relative proportions of total T-cells (i.e., positive for the CD3 cell-surface marker) and helper T-cells (i.e., positive for the CD3 and CD4 cell-surface markers) did not differ between uninfected and infected control animals. However, PRRSV-infection increased ($P < 0.05$) the relative proportion of cytotoxic T-cells (i.e., positive for the CD3 and CD8 cell-surface markers) in the total lymphocyte population when compared with uninfected pigs receiving the Control diet. PRRSV-infection also increased ($P < 0.05$) the proportion of dual-positive T-cells

(i.e., positive for the CD3, CD4, and CD8 cell-surface markers), but showed a reduction in the percentage of dual-positive T-cells with active antiviral effector pathways (i.e., dual-positive T-cells also expressing intracellular maker IFN-γ).

Among PRRSV-infected pigs, there was a main effect ($P = 0.011$) of ISF supplementation on the relative proportion of helper T-cell populations, with ISF supplementation increasing ($P < 0.05$) the proportion of helper T-cells (**Figure 3.3**). PRRSV-infected pigs receiving the ETSBM + ISF diet maintained the largest proportion of helper T-cells (26.4%), which was higher than pigs receiving the Control or ETSBM diets without supplemental ISF. Pigs receiving the Control + ISF diet maintained the second largest proportion, which was not different from other treatment groups. Across all immunophenotyping parameters, there were no main effects of soy source or interaction between soy source and ISF supplementation.

Discussion

Isoflavones are bioactive components that have been shown to have antiviral activity and are found in high concentrations in soybeans and soybean-derived feedstuffs (Wang and Murphy, 1996). This activity has been demonstrated against PRRSV specifically in live animal studies previously (Greiner et al., 2001a; Greiner et al., 2001b; Rochell et al., 2015). However, these studies were unable to identify the effects of soy ISF when present in normal concentrations and proportions found naturally in soybean meal without confounding effects of differing amino acid concentrations or sources in the diet. For that reason, one of our aims was to determine effects of industry relevant concentrations of soy ISF in diets otherwise devoid of these compounds and balanced for SID amino acid concentrations on the response of pigs to disease challenge. The two protein sources used for this study were a soy protein concentrate

(**SPC**; Arcon AF, ADM, Decatur, IL) and enzyme-treated soybean meal (**ETSBM**; HP300, Hamlet Protein, Findlay, OH), both of which are soy protein sources that have undergone additional processing beyond that of SBM. Soybean meal, while an affordable protein option, is often limited in weanling pig diets for the first few weeks post-weaning due to the immaturity of the weanling pig's digestive capacity and presence of antinutritional factors such as oligosaccharides that can exacerbate gastrointestinal distress in young pigs (Stein, 2002). However, additional processing of SBM to either SPC or ETSBM can remove some of these antinutritional factors by solubilizing and removing residual carbohydrate fractions (Peisker, 2001). Soy protein concentrate typically contains larger residual carbohydrate fractions than soy protein isolate, but both will have a greater proportions of protein than SBM (Shurtleff and Aoyagi, 2016). It is important to note that during the manufacturing of SPC and SPI, much of the estrogenic and antigenic factors such as soy ISF are also removed, which allowed us to develop basal diets with minimal to no ISF present. Using protein sources largely devoid of ISF ensured that we could supplement soy-derived ISF directly using a highly enriched source to reach concentrations near that of what is normally observed in SBM at typical inclusion levels for a nursery swine diet (USDA-ARS, 2016).

In general, our disease challenge model reduced ADG and growth efficiency of infected individuals, indicating that a successful infection with PRRSV was established. This was also supported by increased rectal temperatures in our infected pigs over that of our non-infected controls throughout the post-inoculation period, which is expected during acute PRRSV infections. Reductions in ADG supported previous findings of foundational PRRSV-challenge studies performed by our laboratory and others, though we observed a more severe reduction in performance than expected (Greiner et al., 2000; Rochell et al., 2015), likely due to differences

in PRRSV strain virulence between studies. Additionally, the presence of an unintended Mh coinfection may have further diverted available energy sources away from body weight gain in our pigs, exacerbating the negative effects of our PRRSV intervention. To the contrary, we did not observe reductions in feed intake following PRRSV-inoculation, which was not in agreement with the findings by Greiner et al. who observed an approximate 22% decrease in ADFI from the 4 d immediately preceding inoculation to days 4 to 8 post-inoculation (Greiner et al., 2000). When determining potential causes for this difference, we should note that there was observable feed wasting by the pigs as they transitioned onto their pelleted diet post-weaning. With that in mind, an improved G:F ratio was observed over the entire growth period from 0 to 14 DPI for pigs fed ETSBM as a protein source. This could be due to the additional processing that ETSBM undergoes as a soy protein product, resulting in greater removal of residual antinutritional factors (e.g., raffinose and stachyose) that may contribute to higher digestibility values of the total mixed diet (Peisker M, 2001).

Aside from reduction in growth performance, other common indicators of infection status in swine respiratory models are serum viral load and circulating inflammatory cytokine concentrations. In previous PRRSV-challenge models, PRRSV infection caused rapid increases in serum viral concentrations when pigs were experimentally inoculated at 29 d of age, peaking at 4 DPI (Greiner et al., 2000). In subsequent studies by the same group evaluating the effects of individual soy ISF supplementation, similar results were observed with serum viral concentrations peaking at 4 DPI (Greiner et al., 2001a; Greiner et al., 2001b). When weaning age pigs were supplemented with soy genistein alone, the primary ISF found in soybeans, a linear reduction of serum PRRS viral concentrations was observed (Greiner et al., 2001a). However, when supplemented with daidzein alone, the second most prevalent ISF found in soybeans, no

effects on serum PRRS viral concentrations were observed, suggesting that other ISF fractions or interactions between ISF fractions possess more antiviral activity (Greiner et al., 2001b; Dia et al., 2008).

Although no significant main effects for soy source were observed in our study, ISF supplementation increased serum PRRS viral concentrations on 3 DPI. A strong interaction between soy source and ISF supplementation was observed, which was most evident in our ETSBM + ISF treatment group that maintained the highest serum PRRS viral concentrations (i.e., lowest Ct values) at both 3 and 6 DPI. In a previous study from our laboratory, concentration of SBM (17.5% vs. 29.0%) in the diet had no influence on Ct values until 14 DPI with pigs fed higher concentrations of SBM having lower serum PRRS viral concentrations (Rochell et al., 2015). Importantly, the methods used to detect serum PRRSV viral antigen differ between the studies discussed above; studies by Greiner et al. utilized a cytopathic effect assay, an indirect viral detection assay involving serial dilutions of serum and viral clearing results of the assay to calculate the virus concentration (Reed and Muench, 1938; Greiner et al., 2000; Greiner et al., 2001a; Greiner et al., 2001b). Rochell et al. (2015) utilized detection of PRRS viral mRNA via real-time PCR, a direct viral detection assay and the same assay utilized in our present study.

Understanding the differences in effects on serum PRRS viral concentrations across previous studies and ours is difficult, mainly due to lack of *in vivo* studies evaluating antiviral properties of ISF. *In vitro*, genistein in particular exhibits high inhibitory activity against viruses. Against human rotavirus, viral infectivity of cultured macrophages (MA-104 cell line) exposed to genistein or a mixture of ISF was reduced by 33% to 72%. Mixtures where genistein was absent lost these reductions of viral infectivity, demonstrating its importance for mediating viral

infections (Andres et al., 2007). Against herpes simplex virus types-1 and -2 (**HSV-1** and **HSV-2**), genistein moderately inhibited cytopathic effects (**CPE**), which are structural changes to the host cell that occur when they become infected by a virus resulting in subsequent cell death (Lyu et al., 2005). As stated above, measuring CPE was how Greiner et al. detected the presence of PRRS virus in serum and observed significant reductions with genistein supplementation utilizing that assay. Therefore, reasoning for why Rochell et al. (2015) and our study did not see similar reductions in serum PRRS viral concentrations may likely be due to our use of real-time PCR vs. a CPE assay, which may be influenced by the presence of free, bio-activated genistein in circulating serum collected for the assay instead of direct effects on the virus itself *in vivo*. Differences in PRRSV strain and severity of established infection (i.e., presence or absence of co-infection and strain virulence factors) may have also affected the ability of ISF to influence the response to PRRSV infections across these studies.

Regarding inflammatory cytokines as an indicator of infection status, previous studies involving PRRSV and ISF mainly focused on IFN-γ, a key pro-inflammatory cytokine released during viral infections. Concentrations of IFN-γ in the current study were well-aligned with what have been observed previously in our laboratory (Rochell et al., 2015), yet we observed here an unremarkable IFN-γ response to dietary ISF supplementation. In the study conducted by Greiner et al. (2000), PRRSV infection alone caused an increase in serum IFN-γ concentrations, peaking at 4 DPI. These researchers suggested that the increase was due to IFN-γ production by cytotoxic T-cells and natural killer cells in response to the virus in order to support clearance of PRRSV by tissue resident macrophages. Subsequent PRRSV-challenge studies by the same group suggested a quadratic reduction of serum IFN-γ concentrations in response to genistein supplementation (Greiner et al., 2001a). Alternatively, daidzein supplementation at 200 or 800 ppm (but not

400 ppm) caused increased serum IFN-γ concentrations at peak viremia (4 DPI) (Greiner et al., 2001b). Researchers speculated that the reductions for genistein alone may be related to its influence on intracellular signaling pathways. Such pathways on which ISF may elicit an inhibitory action are mainly initiated by tyrosine-specific kinases, which are also targets for a variety of virus types though this is not the case for PRRSV. However, inhibition of these kinases is likely a primary mechanism for reducing viral infectivity by ISF (Akiyama et al., 1987). In addition to tyrosine kinase mediation, genistein administered at low concentrations (0.5–5 µM) may stimulate natural killer cell activity *in vitro*, which could increase the rate of pathogen clearance (Zhang et al., 1999). Within our study, minimal effects on IFN-γ concentrations were observed, suggesting that ISF at the concentrations provided in study only mildly influenced cellular pathways involved in IFN-γ production.

Under the influence of inflammatory cytokines, erythrocyte and leukocyte production and systemic concentrations are also affected by disease status. By 14 DPI, PRRSV-infection decreased RBC counts and elicited reductions in associated parameters (e.g., HGB, HCT, MCV, and MCH). There is evidence that PRRSV-infection and virulence factors associated with specific strains contribute to the onset of anemia with varying severity (Halbur et al., 2002). Although previous research suggests that diets containing higher concentrations of SBM had improved hematocrit values and tendency for greater hemoglobin concentrations, both of which are used as clinical indicators of anemia severity, our findings do not suggest that soy source or ISF supplementation plays a factor in those pathways (Rochell et al., 2015). Regarding differential WBC counts, PRRSV-infection caused leukopenia detectable starting at 6 DPI, which is consistent with previous PRRSV studies (Halbur et al., 2002; Toepfer-Berg et al., 2004; Liu et al., 2013). For our study, ISF supplementation within soy source decreased relative band

neutrophil (i.e., immature neutrophil) populations during the acute phase of infection. This could suggest that ISF reduces the stimulation of aggressive pro-inflammatory pathways in the early immune response to PRRSV, which may confer benefits to the overall health and recovery of the pig.

Regarding other measures of inflammatory status, immunophenotyping results by flow cytometry indicated that PRRSV in the presence of a Mh co-infection resulted in shifts of peripheral effector T-cell populations. Effector T-cells have specific and complementary effector functions that aid in the clearance of pathogens, but alterations of proportions of these cells may indicate disruptions in immune system function. In the human medical field, the ratio of effector helper T-cell and cytotoxic T-cell populations has been utilized as a measure of immune system status and presence of immunosuppression. Although not well characterized among healthy individuals, the ratio of helper T-cells to cytotoxic T-cells (CD4:CD8 or helper/suppressor to cytotoxic) typically ranges between 1.5 and 2.0 with a higher ratio value indicating better immune system status. An inverse of this ratio can result by targeted cell death of helper T-cells, population expansion of cytotoxic T-cells, or a combination thereof (Mcbride and Striker, 2017). In humans, this ratio tends to decrease as they age, a phenomenon referred to as "immunosenesence," and humans of older age groups with inverted CD4:CD8 ratios had higher mortality rates than those with normal ratios (Wikby et al., 2005).

For the pigs in this study, PRRSV-infection appeared to inverse the CD4:CD8 ratio by increasing the proportion of cytotoxic T-cells in the periphery. For PRRSV-infected pigs fed the Control diet, infection resulted in a reduction of the CD4:CD8 ratio by half (2.15 vs. 1.06 for PRRSV-infected vs. non-infected animals receiving the Control diet, respectively). Within our PRRSV-infected pigs and independent of soy source, consumption of ISF increased the

CD4:CD8 T-cell ratio from 0.99 to 1.35 (**Figure 3.3**). We also observed an increased proportion of peripheral helper T-cells, which partially restored the CD4:CD8 ratio to be closer to that observed in non-infected control animals. Although there is a lack of information on whether this relationship between helper and cytotoxic T-cells is also observed in swine, it could suggest that increased efforts to better understand implications of altered ratios of these effector cell populations on susceptibility to disease in growing pigs may be beneficial.

Implications

When isolating the impact of ISF on disease-challenged pigs, they appear to be non-beneficial regarding growth performance. However, adaptive immune responses appear to be altered beneficially when disease-challenged growing pigs consume ISF, which may elicit benefits during the recovery period in terms of compensatory growth. For these reasons, studying the effect of soy ISF throughout the grow–finish period is warranted.

Tables and Figures

Table 3.1. Experimental treatments[1]

Treatment	Dietary treatment	Infection status[2]
Control	Soy protein concentrate	Uninfected
Control	Soy protein concentrate	PRRSV-infected
Control + ISF	Soy protein concentrate + ISF	PRRSV-infected
ETSBM[3]	ETSBM	PRRSV-infected
ETSBM[3] + ISF	ETSBM + ISF	PRRSV-infected

[1]Abbreviations: ISF, soy isoflavones; PRRSV, porcine reproductive and respiratory syndrome virus, Mh, *Mycoplasma hyopneumoniae*.
[2]All pigs were naturally co-infected with Mh prior to the start of the study at the source farm.
[3]Enzyme-treated soybean meal (ETSBM) manufactured to contain a gentle soya-yeast (10% yeast components) supplement for piglet feed with a low content of anti-nutritional factors (trypsin inhibitors, antigens, and flatulent oligosaccharides); Hamlet Protein, Findlay, OH.

Table 3.2. Analyzed isoflavone, saponin, and amino acid concentrations of experimental ingredients (as-fed basis)[1]

	Ingredient		
Item	**SPC[2]**	**ETSBM[3]**	**ISF[4]**
Isoflavones, mg/kg			
Total genistein	0.00	908	177,317
Total daidzein	0.00	1,314	201,902
Total glycitein	0.00	196	22,983
Total isoflavones	0.00	2,417	402,203
Total saponins, mg/kg	1,313	3,656	-
Total AA[4]			
Indispensable AA[4], g/kg			
Arginine	4.89	3.91	0.12
Histidine	1.71	1.36	0.04
Isoleucine	3.29	2.54	0.06
Leucine	5.28	4.14	0.07
Lysine	4.30	3.29	0.06
Methionine	0.92	0.73	0.02
Phenylalanine	3.44	2.74	0.20
Threonine	2.55	2.09	0.05
Tryptophan	0.96	0.75	0.33
Valine	3.41	2.65	0.06
Dispensable AA[4], g/kg			
Alanine	2.84	2.34	0.07
Aspartic acid	7.43	5.97	0.20
Cysteine	0.89	0.72	0.07
Glutamic acid	12.41	9.80	0.34
Glycine	2.83	2.28	0.08
Proline	3.50	2.76	0.12
Serine	2.92	2.46	0.04
Tyrosine	2.32	1.95	0.17

[1]Abbreviations: SPC, soy protein concentrate; ETSBM, enzyme-treated soybean meal; ISF, isoflavones; AA, amino acids.
[2]SPC manufactured by traditional process to remove soluble sugars and reduce anti-nutritional factors; ADM Foods & Wellness, Decatur, IL.
[3] ETSBM manufactured to contain a gentle soya-yeast (10% yeast components) supplement for piglet feed with a low content of anti-nutritional factors (trypsin inhibitors, antigens, and flatulent oligosaccharides); Hamlet Protein, Findlay, OH.
[4] ISF provided by Novasoy 400, which has a guaranteed 40% minimum total isoflavone concentration (referred to as suppl. ISF in subsequent tables); ADM, Decatur, IL. Diets were formulated to contain ISF concentrations similar to those observed in typical weaning commercial diets containing SBM.

Table 3.3. Ingredient and calculated composition of experimental diets (as-fed basis)[1]

	Soy source	**SPC**		**ETSBM**	
Item	***Suppl. ISF***	**No**	**Yes**	**No**	**Yes**
Ingredient, %					
Corn		62.52	62.11	59.25	58.91
HP300		0.00	0.00	20.80	20.80
Arcon AF		17.50	17.50	0.00	0.00
Dried whey		12.00	12.00	12.00	12.00
Poultry by-product meal[2]		4.00	4.00	4.00	4.00
Choice white grease		1.50	1.50	1.50	1.50
Ground limestone		0.60	0.60	0.60	0.60
Monocalcium phosphate		0.20	0.20	0.20	0.20
Sodium chloride		0.40	0.40	0.40	0.40
Vitamin and mineral premix[3]		0.30	0.30	0.30	0.30
Choline chloride		0.07	0.07	0.07	0.07
L-Lys HCl		0.43	0.43	0.49	0.49
DL-Met		0.18	0.18	0.16	0.16
L-Trp		0.07	0.07	0.05	0.05
L-Thr		0.20	0.20	0.18	0.18
L-Val		0.03	0.03	0.00	0.00
Novasoy400		0.00	0.41	0.00	0.34
Calculated composition					
ME, kcal/kg[4]		3,472	3,458	3,465	3,453
CP, %[4]		21.29	21.26	21.28	21.25
SID AA, %[4]					
Lysine		1.34	1.34	1.34	1.34
Methionine + Cysteine		0.73	0.73	0.74	0.74
Tryptophan		0.26	0.26	0.26	0.26
Threonine		0.86	0.86	0.86	0.86
Valine		0.88	0.88	0.88	0.88
Isoflavones, mg/kg					
Total genistein		2	690	135	702
Total daidzein		1	723	105	701
Total glycitein		0	87	25	97
Total isoflavones		3	1,500	265	1,500
Total saponins, mg/kg		664	1,013	840	1,128

[1]All pigs received allotted treatment diet upon starting -7 DPI. Abbreviations: DPI, day post-inoculation; ISF, isoflavones; SID, standardized ileal digestible, AA, amino acids.

[2]Low ash pet-food-grade poultry by-product meal, American Proteins, Inc., Hanceville, AL

[3]Vitamin-mineral premix (JBS United, Sheradin, IN) included the following per kilogram of complete diet: Vitamin A (retinyl acetate), 11,128 IU; Vitamin D3 (cholecalciferol), 2,204 IU; Vitamin E (dl-α tocopheryl acetate), 66 IU; Vitamin K (menadione nicotinamide bisulfite), 1.42 mg; Thiamine (thiamine mononitrate), 0.24 mg; Riboflavin, 6.58 mg; Pyridoxine (pyridoxine hydrochloride), 0.24 mg; Vitamin B12, 0.03 mg; d-Pantothenic acid (d-calcium pantothenate), 23.5 mg; Niacin (nicotinamide and nicotinic acid), 44 mg; Folic acid, 1.58 mg; Biotin, 0.44 mg; Cu (copper sulfate), 10 mg; Fe (iron sulfate), 125 mg; I (potassium iodate), 1.26 mg; Mn (manganese sulfate), 60 mg; Se (sodium selenite), 0.3 mg; and Zn (zinc oxide), 100 mg.

[4]Metablizable energy and standardized ileal digestible (SID) amino acid values were calculated using NRC (2012). Analyzed crude protein determined as CP = (N × 6.25).

Table 3.4. Analyzed composition of experimental diets (as-fed basis)[1]

	Soy source	**SPC**		**ETSBM**	
Item	***Suppl. ISF***	**No**	**Yes**	**No**	**Yes**
Dry matter, %		90.66	90.19	90.26	90.03
Organic matter, %		94.60	94.42	94.30	94.19
Crude protein[2], %		21.29	21.26	21.28	21.25
Lactose, %		8.75	8.75	8.75	8.75
Total dietary fiber, %		11.89	11.83	11.83	11.79
Isoflavones, mg/kg					
Total genistein		52.0	757	291	947
Total daidzein		0.00	625	358	969
Total glycitein		34.0	69.0	48.0	125
Total isoflavones		87.0	1,451	697	2,041
Total saponins, mg/kg		1,336	1,316	1,382	1,252
Total AA					
Indispensable AA, g/kg					
Arginine		12.8	12.6	12.0	12.4
Histidine		5.00	5.00	4.80	4.90
Isoleucine		9.10	9.00	8.80	9.20
Leucine		17.5	17.3	16.9	17.2
Lysine		14.8	14.4	14.2	14.8
Methionine		4.80	4.70	4.20	4.50
Phenylalanine		9.70	9.50	9.40	9.70
Threonine		9.90	9.60	9.10	9.70
Tryptophan		3.10	3.00	2.80	3.00
Valine		10.4	10.4	9.80	10.2
Dispensable AA, g/kg					
Alanine		10.6	10.4	10.0	10.4
Aspartic acid		19.7	19.5	18.7	19.5
Cysteine		3.00	3.00	2.90	2.90
Glutamic acid		35.4	35.3	34.3	34.9
Glycine		9.80	9.40	8.90	9.50
Proline		12.2	11.9	11.4	11.9
Serine		8.70	8.70	8.30	8.50
Tyrosine		6.50	6.40	6.10	6.50

[1]All pigs received allotted treatment diet upon starting -7 DPI. Abbreviations: DPI, day post-inoculation; ISF, isoflavones, AA, amino acids.
[2]Analyzed crude protein determined as % total nitrogen × 6.25.

Table 3.5. Effects of dietary soy isoflavones level and porcine reproductive and respiratory virus (PRRSV) infection on growth performance of weanling pigs[1]

							P-value			
	Uninfected	PRRSV-infected						Main effects[3]		Interaction
Item	Control[2]	Control[2]	Control + ISF[2]	ETSBM[2]	ETSBM + ISF[2]	SEM	Uninfected vs. Infected Control	Soy source	ISF	Soy Source × ISF
BW, kg										
initial	5.72	5.74	5.57	5.73	5.70	0.43	0.872	0.887	0.814	0.992
final	12.12	8.65*	7.89	9.28	8.91	0.45	< 0.0001	0.057	0.196	0.151
-7 to 0 DPI[4]										
ADG, g/d	161	127	160	155	173	20.9	0.287	0.321	0.214	0.727
ADFI, g/d[5]	677	632*	654	604	649	21.6	0.042	0.453	0.137	0.621
G:F, g/kg[5]	264	214	240	259	275	35.1	0.300	0.272	0.559	0.878
0 to 6 DPI										
ADG, g/d	275	103*	83.2	126	107	35.2	0.0001	0.362	0.441	0.965
ADFI, g/d[5]	1,236	1,381	1,188	1,128	1,403	257.0	0.295	0.885	0.756	0.084
G:F, g/kg[5]	248	75.5*	99.2	120	93.6	37.1	< 0.0001	0.551	0.977	0.443
6 to 14 DPI										
ADG, g/d	460	190*	156	270	219	46.7	0.0002	0.099	0.317	0.838
ADFI, g/d[5]	728	489	423	549	450	149.8	0.080	0.633	0.442	0.845
G:F, g/kg[5]	526	437	268	484	173	99.5	0.384	0.803	0.020	0.463
0 to 14 DPI[4]										
ADG, g/d	380	137*	103	219	165	39.5	< 0.0001	0.015	0.125	0.712
ADFI, g/d[5]	775	648	558	629	601	186.6	0.229	0.888	0.485	0.708
G:F, g/kg[5]	566	250*	171	378	361	59.8	0.0001	0.009	0.413	0.598

*Difference ($P < 0.05$) between uninfected and infected groups fed the control diet.

[1]Values represent least square means of 6 to 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI = days post-inoculation; ISF = isoflavones.

[2]The following diet names have been assigned to a respective experimental diet groups: *Control*: soy protein concentrate + no supplemented ISF, fed to both uninfected and PRRSV-infected control pigs; *Control + ISF*: soy protein concentrate + supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM*: ETSBM + no supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM + ISF*: ETSBM + supplemented ISF, fed to PRRSV-infected pigs only.

[3]Main effect of day post-inoculation (DPI); no 2- or 3-way interactive effects involving DPI were significant, so they were not included in the statistical model.

[4]Feed intake data and G:F values for -7-0 DPI from the first cohort of pigs (n=28) were omitted due to a mechanical error with the feeders that led to incorrect feed intake measurements.

[5]ADFI was omitted if there was a net weight loss over measured period; G:F data was omitted if calculated value was less than 0 g/kg or greater than 1,000 g/kg

Table 3.6. Effects of dietary soy isoflavones level and porcine reproductive and respiratory virus (PRRSV) infection on daily rectal temperatures (ºC) of weanling pigs[1]

							P-value			
	Uninfected	PRRSV-infected						Main effects[3]		Interaction
DPI	Control[2]	Control[2]	Control + ISF[2]	ETSBM[2]	ETSBM + ISF[2]	SEM	Uninfected vs. Infected Control	Soy Source	ISF	Soy Source × ISF
0	39.36	39.86*	39.91	39.69	39.65	0.28	0.036	0.228	0.971	0.681
3	39.48	40.07*	39.92	40.35	40.11	0.27	0.012	0.197	0.266	0.400
6	39.65	40.12	40.42	40.47	40.18	0.27	0.051	0.748	0.965	0.399
8	39.58	40.36*	40.69	40.50	40.62	0.27	0.001	0.848	0.205	0.562
12	40.01	40.24	40.68	40.50	40.49	0.27	0.340	0.855	0.247	0.417
14	39.88	40.29	40.21	40.29	40.40	0.27	0.091	0.630	0.949	0.922

*Difference ($P < 0.05$) between uninfected and infected groups fed the control diet.

[1]Values represent least square means of 10 to 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI = days post-inoculation; ISF = isoflavones.

[2]The following diet names have been assigned to a respective experimental diet groups: *Control*: soy protein concentrate + no supplemented ISF, fed to both uninfected and PRRSV-infected control pigs; *Control + ISF*: soy protein concentrate + supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM*: ETSBM + no supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM + ISF*: ETSBM + supplemented ISF, fed to PRRSV-infected pigs only.

[3]Main effect of day post-inoculation (DPI); no 2- or 3-way interactive effects involving DPI were significant, so they were not included in the statistical model.

Table 3.7. Effects of dietary soy isoflavones level and porcine reproductive and respiratory virus (PRRSV) infection on serum viral load and presence of anti-PRRSV antibodies in weanling pigs[1]

							P-value		
	Uninfected	PRRSV-infected					Main effects[3]		Interaction
Item	Control[2]	Control[2]	Control + ISF[2]	ETSBM[2]	ETSBM + ISF[2]	SEM	Uninfected vs. Infected Control	Soy Source	ISF	Soy Source × ISF
Ct Value[4]										
0 DPI	ND	ND	ND	ND	ND	1.52	0.812	0.968	0.968	0.999
3 DPI	ND	15.57[*b]	16.44[b]	18.81[b]	12.10[a]	1.41	< 0.001	0.592	0.005	0.0001
6 DPI	ND	14.12[*]	15.39	15.37	12.66	1.41	< 0.001	0.522	0.430	0.166
14 DPI	ND	18.75[*]	19.09	19.51	18.69	1.45	< 0.001	0.860	0.795	0.948
ELISA S/P Ratio[5]										
6 DPI	--	0.087	0.070	0.040	0.045	0.10	--	0.661	0.935	0.974
14 DPI	--	1.565	1.736	1.475	1.440	0.171	--	0.027	0.412	0.080

[*]Difference ($P < 0.05$) between uninfected and infected groups fed the control diet. [ab]Means without common superscript letter do differ ($P < 0.05$).

[1]Values represent least square means of 10 to 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI = days post-inoculation; ISF = isoflavones; ELISA = enzyme-linked immunosorbent assay.

[2]The following diet names have been assigned to a respective experimental diet groups: *Control*: soy protein concentrate + no supplemented ISF, fed to both uninfected and PRRSV-infected control pigs; *Control + ISF*: soy protein concentrate + supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM*: ETSBM + no supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM + ISF*: ETSBM + supplemented ISF, fed to PRRSV-infected pigs only.

[3]Main effect of day post-inoculation (DPI); no 2- or 3-way interactive effects involving DPI were significant, so they were not included in the statistical model.

[4]Cycle threshold (Ct) values represent the mean number of PCR cycles required to detect the presence of PRRSV mRNA. A higher Ct value indicates less PRRSV RNA in the serum. No PRRSV DNA was detected in the serum of any uninfected pigs.

[5]IDEXX-ELISA S/P Ratio of 0.4 or greater is considered positive result. IDEXX ELISA assays were not performed on samples confirmed negative by qRT-PCR.

Table 3.8. Effects of dietary soy isoflavone levels and porcine reproductive and respiratory virus (PRRSV) infection on red blood cell measures in weanling pigs[1]

							P-value			
	Uninfected	PRRSV-infected						Main effects[3]		Interaction
Item	Control[2]	Control[2]	Control + ISF[2]	ETSBM[2]	ETSBM + ISF[2]	SEM	Uninfected vs. Infected Control	Soy Source	ISF	Soy Source × ISF
RBC, × 10^6 cells/µL										
0 DPI	6.53	6.66	6.84	6.49	6.81	0.180	0.624	0.456	0.099	0.305
3 DPI	6.20	6.00	6.14	5.90	6.29	0.180	0.419	0.862	0.066	0.234
6 DPI	5.97	6.05	6.17	5.87	6.00	0.175	0.778	0.220	0.375	0.502
14 DPI	5.85	5.31*	5.58	5.25	5.43	0.187	0.050	0.519	0.129	0.436
HGB, g/dL										
0 DPI	12.3	11.9	12.5	12.2	12.5	0.341	0.552	0.750	0.190	0.588
3 DPI	11.5	10.6	11.1	10.8	11.4	0.341	0.119	0.472	0.070	0.268
6 DPI	10.9	10.4	10.9	10.5	10.8	0.326	0.386	0.888	0.205	0.633
14 DPI	11.0	9.0*	9.5	9.1	9.5	0.358	0.002	0.874	0.156	0.540
HCT, %										
0 DPI	35.1	37.4	39.1	37.9	39.1	0.976	0.294	0.882	0.172	0.591
3 DPI	35.7	33.4	34.9	33.8	35.6	0.976	0.272	0.537	0.081	0.314
6 DPI	34.0	32.9	34.4	33.1	33.7	0.930	0.635	0.722	0.301	0.708
14 DPI	34.7	28.5*	30.3	28.8	30.0	1.03	0.010	0.893	0.116	0.442
MCV, fl										
0 DPI	58.6	56.2	57.2	58.5	57.5	1.91	0.281	0.257	0.937	0.579
3 DPI	57.9	55.7	56.9	57.3	56.7	1.75	0.300	0.500	0.801	0.742
6 DPI	57.4	54.6	55.8	56.4	56.1	1.72	0.209	0.346	0.712	0.706
14 DPI	59.6	53.7*	54.2	55.0	55.3	1.79	0.013	0.292	0.700	0.719

*Difference ($P < 0.05$) between uninfected and infected groups fed the control diet. [ab]Means without common superscript letter do differ ($P < 0.05$).

[1]Values represent least square means of 10 to 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI = days post-inoculation; ISF = isoflavones; RBC = red blood cells; HGB = hemoglobin; HCT = packed cell volume; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration

[2]The following diet names have been assigned to a respective experimental diet groups: *Control*: soy protein concentrate + no supplemented ISF, fed to both uninfected and PRRSV-infected control pigs; *Control + ISF*: soy protein concentrate + supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM*: ETSBM + no supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM + ISF*: ETSBM + supplemented ISF, fed to PRRSV-infected pigs only.

[3]2- or 3-way interactive effects involving DPI were significant, so they were not included in the statistical model.

Table 3.8. Continued[1]

							P-value			
	Uninfected	PRRSV-infected						Main effects[3]		Interaction
Item	Control[2]	Control[2]	Control + ISF[2]	ETSBM[2]	ETSBM + ISF[2]	SEM	Uninfected vs. Infected Control	Soy Source	ISF	Soy Source × ISF
MCH, pg										
0 DPI	18.6	17.9	18.3	18.8	18.4	0.662	0.331	0.224	0.961	0.498
3 DPI	18.4	17.7	18.1	18.3	18.1	0.650	0.374	0.451	0.698	0.717
6 DPI	18.2	17.3	17.7	17.9	18.0	0.650	0.219	0.283	0.510	0.632
14 DPI	18.8	17.0*	17.1	17.4	17.5	0.675	0.037	0.312	0.775	0.762
MCHC, g/dL										
0 DPI	31.8	31.6	32.0	32.0	32.0	0.734	0.721	0.842	0.871	0.988
3 DPI	31.7	31.6[b]	31.9[b]	31.9[b]	29.3[a]	0.734	0.943	0.092	0.096	0.024
6 DPI	31.7	31.5	31.8	31.6	32.0	0.700	0.561	0.836	0.655	0.968
14 DPI	31.5	31.6	31.6	31.6	31.7	0.774	0.662	0.920	0.991	0.999

*Difference ($P < 0.05$) between uninfected and infected groups fed the control diet. [ab]Means without common superscript letter do differ ($P < 0.05$).

[1]Values represent least square means of 10 to 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI = days post-inoculation; ISF = isoflavones; RBC = red blood cells; HGB = hemoglobin; HCT = packed cell volume; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration

[2]The following diet names have been assigned to a respective experimental diet groups: *Control*: soy protein concentrate + no supplemented ISF, fed to both uninfected and PRRSV-infected control pigs; *Control + ISF*: soy protein concentrate + supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM*: ETSBM + no supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM + ISF*: ETSBM + supplemented ISF, fed to PRRSV-infected pigs only.

[3]Main effect of day post-inoculation (DPI); no 2- or 3-way interactive effects involving DPI were significant, so they were not included in the statistical model.

Table 3.9. Effects of dietary soy isoflavones level and porcine reproductive and respiratory virus (PRRSV) infection on differential leukocyte counts and relative population proportions in weanling pigs[1]

							P-value			
	Uninfected	PRRSV-infected						Main effects[3]		Interaction
Item	Control[2]	Control[2]	Control + ISF[2]	ETSBM[2]	ETSBM + ISF[2]	SEM	Uninfected vs. Infected Control	Soy Source	ISF	Soy Source × ISF
WBC, × 10^3 cells/μL										
0 DPI	15.2	13.3	13.4	14.8	17.2	3.39	0.359	0.447	0.719	0.826
3 DPI	16.1	12.2	9.83	14.2	13.7	3.39	0.061	0.360	0.670	0.789
6 DPI	16.4	11.8[*a]	12.7[a]	24.4[b]	14.5[a]	3.24	0.031	0.026	0.153	0.018
14 DPI	16.6	23.1[*]	24.5	23.2	23.5	3.57	0.006	0.929	0.787	0.990
NEU, % of WBC										
0 DPI	49.1	45.3	38.9	47.5	40.5	4.64	0.522	0.671	0.137	0.492
3 DPI	46.3	56.5	57.1	57.8	55.1	4.64	0.076	0.938	0.811	0.977
6 DPI	38.7	51.5[*]	54.2	59.1	47.4	4.42	0.030	0.345	0.301	0.281
14 DPI	35.7	49.9[*]	43.2	46.9	39.4	4.89	0.027	0.466	0.134	0.340
BAND, % of WBC										
0 DPI	0.00	0.135	0.00	0.550	0.333	2.06	0.858	0.810	0.924	0.995
3 DPI	1.10	6.00[*]	1.10	6.63	3.18	2.06	0.038	0.412	0.012	0.073
6 DPI	0.304	6.11[*]	2.45	5.25	5.27	1.99	0.016	0.542	0.267	0.422
14 DPI	0.087	1.27	4.44	1.77	3.63	2.14	0.648	0.922	0.157	0.546
LYM, % of WBC										
0 DPI	47.1	45.7	51.3	45.3	51.7	4.44	0.780	0.973	0.149	0.546
3 DPI	47.1	31.1[*]	35.8	31.0	37.5	4.43	0.002	0.851	0.179	0.588
6 DPI	52.9	32.2[*]	32.8	27.7	38.1	4.24	0.0001	0.905	0.176	0.348
14 DPI	51.7	39.5[*]	41.9	45.0	47.7	4.67	0.032	0.219	0.592	0.588

[*]Difference ($P < 0.05$) between uninfected and infected groups fed the control diet. [ab]Means without common superscript letter do differ ($P < 0.05$).

[1]Values represent least square means of 10 to 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI = days post-inoculation; ISF = isoflavones; WBC = white blood cells; NEU = neutrophils; BAND = immature neutrophils aka. band cells; LYM = lymphocytes; MONO = monocytes; EOSIN = eosinophils; BASO = basophils.

[2]The following diet names have been assigned to a respective experimental diet groups: *Control*: soy protein concentrate + no supplemented ISF, fed to both uninfected and PRRSV-infected control pigs; *Control + ISF*: soy protein concentrate + supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM*: ETSBM + no supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM + ISF*: ETSBM + supplemented ISF, fed to PRRSV-infected pigs only.

[3]Main effect of day post-inoculation (DPI); no 2- or 3-way interactive effects involving DPI were significant, so they were not included in the statistical model.

Table 3.9. Continued[1]

	Uninfected	PRRSV-infected					*P*-value			
								Main effects[3]		Interaction
Item	Control[2]	Control[2]	Control + ISF[2]	ETSBM[2]	ETSBM + ISF[2]	SEM	Uninfected vs. Infected Control	Soy Source	ISF	Soy Source × ISF
MONO, % of WBC										
0 DPI	2.77	7.09[*]	7.19	5.56	6.00	1.22	0.016	0.229	0.842	0.379
3 DPI	4.47	3.25	2.10	2.98	2.62	1.22	0.472	0.911	0.508	0.905
6 DPI	6.06	6.50	7.53	5.42	5.62	1.17	0.804	0.181	0.594	0.530
14 DPI	4.73	4.81	5.86	3.70	5.07	1.28	0.963	0.454	0.307	0.679
EOSIN, % of WBC										
0 DPI	1.19	1.63	2.11	1.14	1.42	0.979	0.644	0.446	0.591	0.830
3 DPI	0.993	2.25[ab]	3.80[b]	1.04[a]	1.31[a]	0.979	0.171	0.013	0.218	0.047
6 DPI	1.79	3.06	2.49	2.25	3.40	0.951	0.176	0.934	0.677	0.669
14 DPI	2.95	4.07	4.15	2.19	3.61	1.01	0.272	0.119	0.358	0.280
BASO, % of WBC										
0 DPI	0.200	0.100	0.207	0.00	0.083	0.240	0.826	0.631	0.682	0.942
3 DPI	0.100	0.917	0.300	0.364	0.182	0.240	0.064	0.144	0.083	0.095
6 DPI	0.200	0.636	0.455	0.250	0.182	0.636	0.327	0.147	0.581	0.482
14 DPI	0.500	0.400	0.261	0.294	0.558	0.252	0.836	0.695	0.798	0.814

[*]Difference ($P < 0.05$) between uninfected and infected groups fed the control diet. [ab]Means without common superscript letter do differ ($P < 0.05$).

[1]Values represent least square means of 10 to 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI = days post-inoculation; ISF = isoflavones; WBC = white blood cells; NEU = neutrophils; BAND = immature neutrophils aka. band cells; LYM = lymphocytes; MONO = monocytes; EOSIN = eosinophils; BASO = basophils.

[2]The following diet names have been assigned to a respective experimental diet groups: *Control*: soy protein concentrate + no supplemented ISF, fed to both uninfected and PRRSV-infected control pigs; *Control + ISF*: soy protein concentrate + supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM*: ETSBM + no supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM + ISF*: ETSBM + supplemented ISF, fed to PRRSV-infected pigs only.

[3]Main effect of day post-inoculation (DPI); no 2- or 3-way interactive effects involving DPI were significant, so they were not included in the statistical model.

Table 3.10. Effects of dietary soy isoflavones level and porcine reproductive and respiratory virus (PRRSV) infection on serum cytokine concentrations (pg/mL) in weanling pigs[1]

							P-value			
	Uninfected	PRRSV-infected						Main effects[3]		Interaction
Item, pg/mL	Control[2]	Control[2]	Control + ISF[2]	ETSBM[2]	ETSBM + ISF[2]	SEM	Uninfected vs. Infected Control	Soy Source	ISF	Soy Source × ISF
TNF-α										
0 DPI	128	33.2	38.7	39.6	50.0	181	0.088	0.953	0.958	0.999
3 DPI	72.7	89.7	120	85.1	104	92.9	0.665	0.897	0.786	0.992
6 DPI	58.7	75.1	140	59.9	73.4	98.8	0.676	0.634	0.666	0.929
14 DPI	52.2	152[*]	113	187	353	92.9	0.017	0.094	0.429	0.160
IFN-α										
0 DPI	18.6	101	202	16.4	134	164	0.722	0.645	0.509	0.882
3 DPI	19.3	1,132[*a]	1,483[ab]	1,643[bc]	2,156[c]	232	< 0.001	0.005	0.039	0.005
6 DPI	142	501	428	529	396	147	0.118	0.990	0.465	0.900
14 DPI	5.31	44.6	17.1	21.6	50.2	190	0.930	0.976	0.998	0.999
IFN-γ										
0 DPI	BDL	BDL	BDL	BDL	BDL	--	--	--	--	--
3 DPI	BDL	39.4[a]	3.39[b]	4.96[b]	3.10[b]	10.1	--	0.069	0.048	0.026
6 DPI	BDL	12.2	8.91	12.2	10.0	9.31	--	0.950	0.746	0.989
14 DPI	2.88	2.58	2.00	2.04	9.62	14.2	0.996	0.772	0.775	0.963
IL-10										
0 DPI	BDL	BDL	12.8	BDL	BDL	39.6	--	--	--	--
3 DPI	BDL	26.2	23.7	33.8	24.9	19.8	--	0.755	0.810	0.977
6 DPI	BDL	27.1	42.1	33.5	31.3	22.9	--	0.914	0.759	0.959
14 DPI	BDL	BDL	33.6	BDL	154	28.0	--	--	--	--

[*]Difference ($P < 0.05$) between uninfected and infected groups fed the control diet. [ab]Means without common superscript letter do differ ($P < 0.05$).

[1]Values represent least square means of 10 to 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI = days post-inoculation; ISF = isoflavones; TNF-α = tumor necrosis factor-α; IFN-α = interferon-α; IFN-γ = interferon-γ; IL-10 = interleukin 10; BDL = below detectable limits.

[2]The following diet names have been assigned to a respective experimental diet groups: *Control*: soy protein concentrate + no supplemented ISF, fed to both uninfected and PRRSV-infected control pigs; *Control + ISF*: soy protein concentrate + supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM*: ETSBM + no supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM + ISF*: ETSBM + supplemented ISF, fed to PRRSV-infected pigs only.

[3]Main effect of day post-inoculation (DPI); no 2- or 3-way interactive effects involving DPI were significant, so they were not included in the statistical model.

Table 3.11. Effects of dietary soy isoflavones level and porcine reproductive and respiratory virus (PRRSV) infection on peripheral blood T cell immunophenotypes of weanling pigs[1]

								P-value		
	Uninfected	PRRSV-infected						Main effects[3]		Interaction
Item	Control[2]	Control[2]	Control + ISF[2]	ETSBM[2]	ETSBM + ISF[2]	SEM	Uninfected vs. Infected Control	Soy Source	ISF	Soy Source × ISF
T-Cell, % (CD3+)[4]	55.30	53.21	53.01	53.89	59.02	6.66	0.610	0.393	0.539	0.434
Helper T-Cell, % (CD3+CD4+)[5]	22.03	20.18	23.09	18.03	26.41	2.13	0.463	0.783	0.011	0.202
Cytotoxic T-Cell, % (CD3+CD8+)[5]	12.44	22.78*	22.43	22.71	19.20	3.21	0.003	0.527	0.459	0.506
Dual-Positive T-Cell, % (CD3+CD4+CD8+)[5]	4.30	6.66*	6.32	6.15	5.13	1.19	0.022	0.287	0.406	0.603
Dual-Positive T-Cell Expressing IFN-γ, % (CD3+CD4+CD8+IFNγ+)[5]	90.10	79.72*	79.25	77.94	83.41	4.06	0.001	0.594	0.292	0.253
Dual-Positive T-Cell Expressing IFN-γ MFI (CD3+CD4+CD8+IFNγ+)[6]	764.1	501.0*	515.4	490.6	577.3	88.5	0.002	0.511	0.212	0.397
CD4:CD8 Ratio	2.15	1.06*	1.17	0.918	1.53	0.176	0.001	0.535	0.049	0.159

*Difference ($P < 0.05$) between uninfected and infected groups fed the control diet.

[1]Values represent least square means of 10 to 12 pigs with collection of blood occurring at 12 DPI. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI = days post-inoculation; ISF = isoflavones.

[2]The following diet names have been assigned to a respective experimental diet groups: *Control*: soy protein concentrate + no supplemented ISF, fed to both uninfected and PRRSV-infected control pigs; *Control + ISF*: soy protein concentrate + supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM*: ETSBM + no supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM + ISF*: ETSBM + supplemented ISF, fed to PRRSV-infected pigs only.

[3]Main effect of day post-inoculation (DPI); no 2- or 3-way interactive effects involving DPI were significant, so they were not included in the statistical model.

[4]Percent of total lymphocytes that are positive for cell-surface marker CD3.

[5]Percent of CD3-positive lymphocytes that are also positive for cell-surface markers CD4, CD8, CD4/CD8, or CD4/CD8 and intercellular IFN-γ

[6]Median fluorescence intensity (MFI) is a measure of the fluorescence intensity in a fluorescence channel being measured. It provides an alternative measurement to percent positive for comparison of cell populations between individuals. It is less sensitive to outliers, which is important for very small cell populations like the dual-positive T-cells.

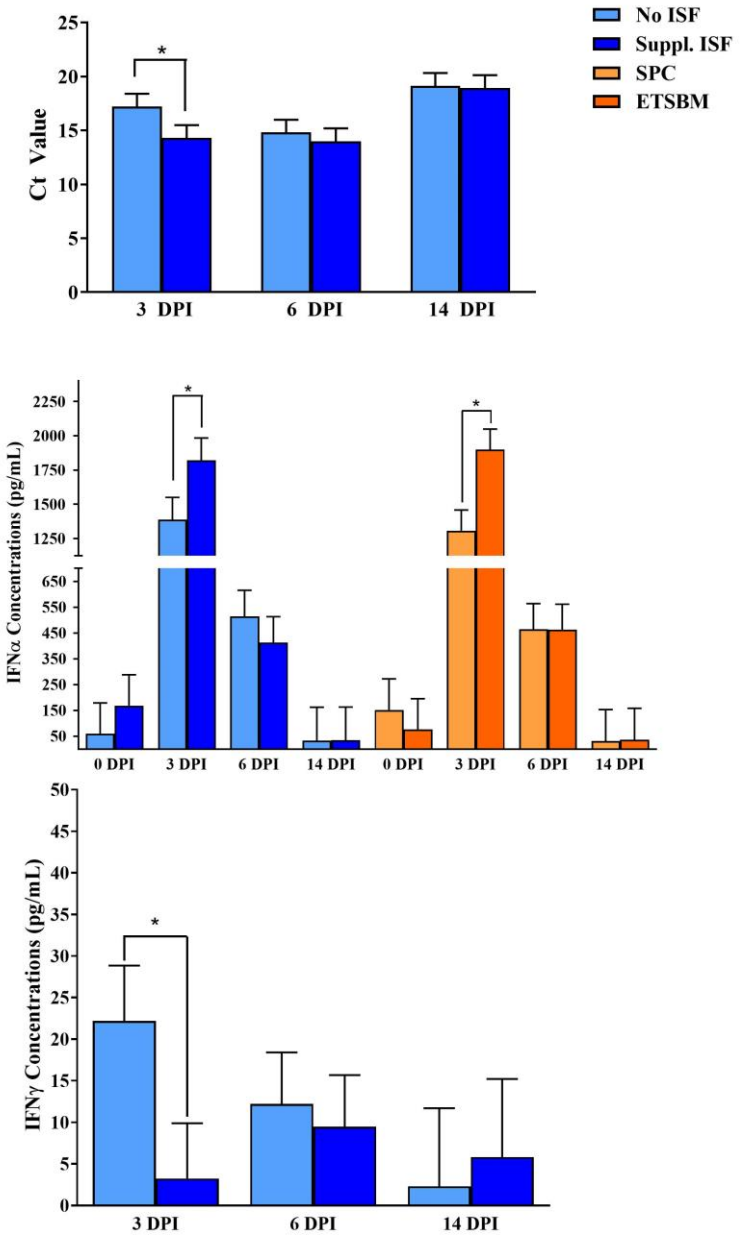

Figure 3.1. Main effect of isoflavone supplementation on the concentration of PRRSV antigen in serum, main effects of both soy source and isoflavone supplementation on serum interferon-α cytokine concentrations, and main effects of isoflavone supplementation on serum interferon-γ cytokine concentrations. PRRSV = porcine reproductive and respiratory syndrome virus; ISF = isoflavones; SPC = soy protein concentrate; ETSBM = enzyme-treated soybean meal; DPI = days post-inoculation; IFN-α = interferon-α; IFN-γ = interferon-γ. Values represent least square means of 20 to 24 pigs, and error bars indicate standard error of the mean. Cycle threshold (Ct) values represent the mean number of PCR cycles required to detect the presence of PRRSV mRNA. A higher Ct value indicates less PRRSV DNA in the serum. An asterisk (*) denotes a difference ($P < 0.05$) due to soy protein source or isoflavone supplementation status at a given time point.

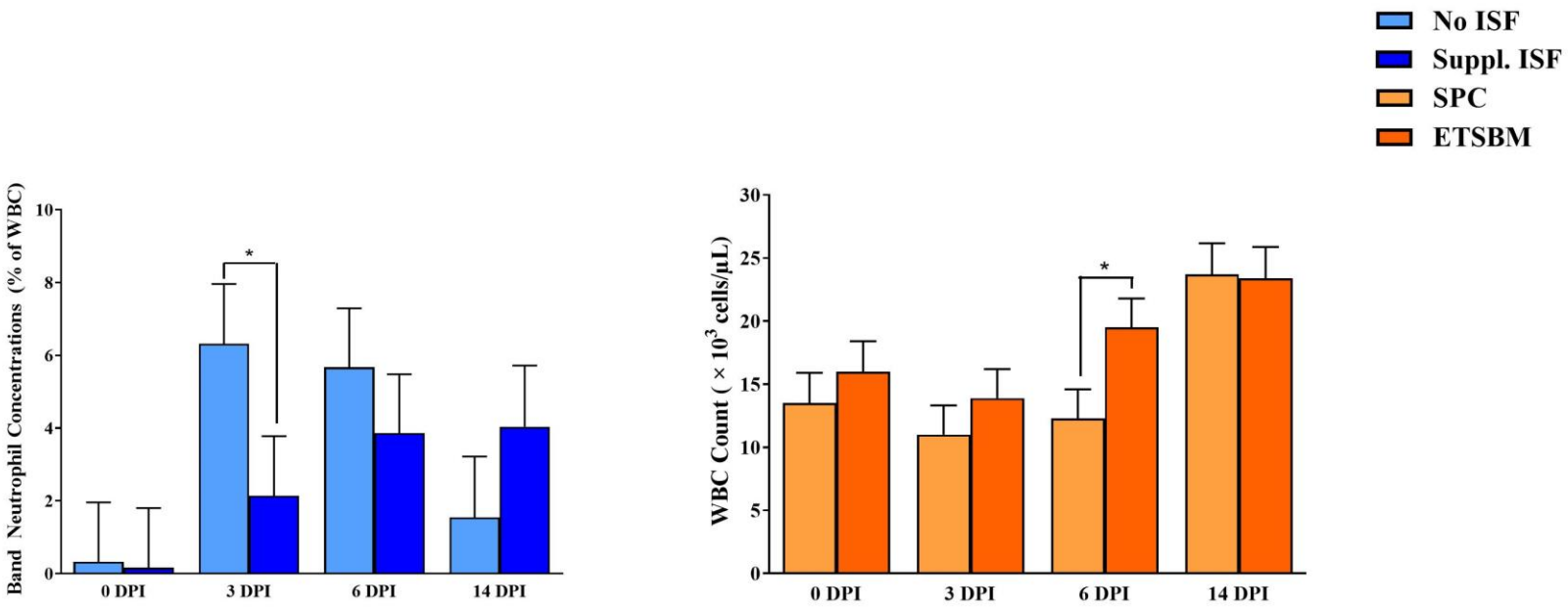

Figure 3.2. Main effect of isoflavone supplementation on the relative concentrations of band neutrophils in whole blood and main effect of soy source on total while blood cell counts in peripheral whole blood. ISF = isoflavones; SPC = soy protein concentrate; ETSBM = enzyme-treated soybean meal; DPI = days post-inoculation; WBC = white blood cells. Values represent least square means of 20 to 24 pigs, and error bars indicate standard error of the mean. An asterisk (*) denotes a difference ($P < 0.05$) due to soy protein source or isoflavone supplementation status at a given time point.

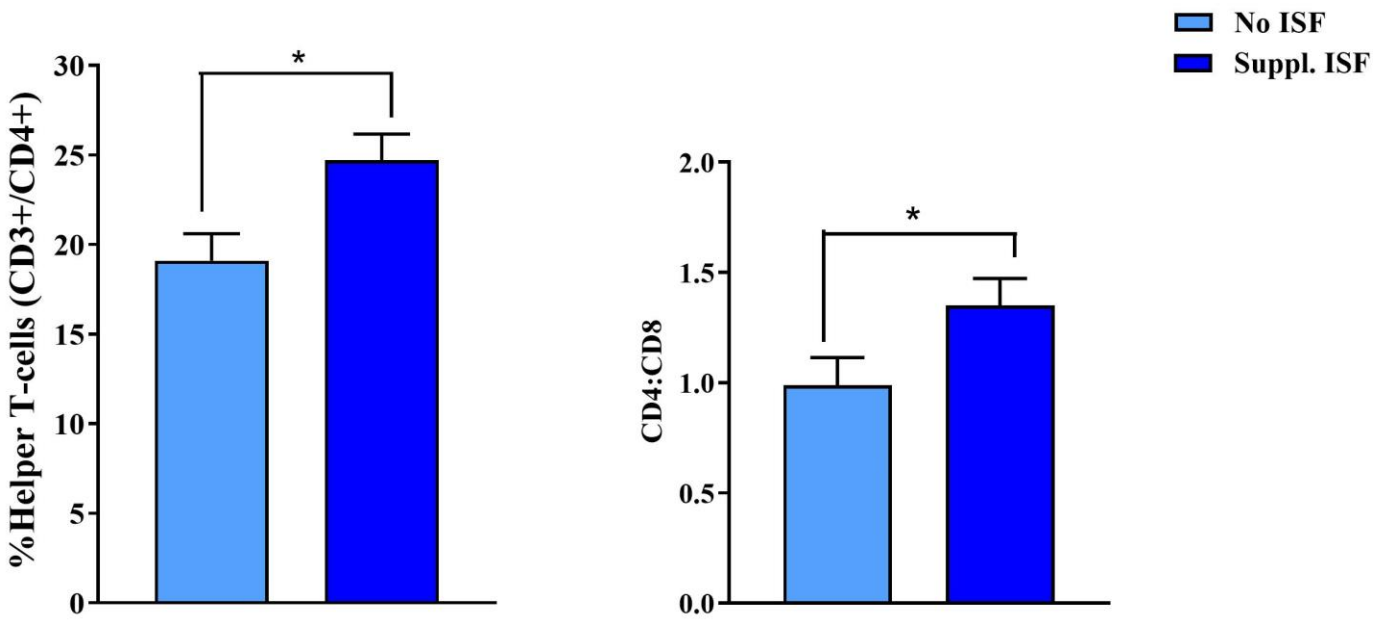

Figure 3.3. Main effects of isoflavone supplementation on the proportion of Helper T-cells (CD3+/CD4+) and Helper:Cytotoxic T-cell ratios (CD4:CD8) in peripheral whole blood. ISF = isoflavones. Values represent least square means of 20 to 24 pigs, and error bars indicate standard error of the mean. An asterisk (*) denotes a difference ($P < 0.05$) due to isoflavone supplementation status for a given outcome.

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CHAPTER 4: DIETARY SOY ISOFLAVONES REDUCE PATHOGEN-RELATED MORTALITY IN GROWING PIGS UNDER PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRAL CHALLENGE[6]

Abstract

Porcine reproductive and respiratory syndrome virus (**PRRSV**) is an economically-important disease and ingestion of soy isoflavones (**ISF**) may benefit PRRSV-infected pigs due to demonstrated anti-inflammatory and anti-viral properties. The objective of this experiment was to recreate immunological effects previously observed in young pigs infected with PRRSV receiving ISF and determine how those effects influence growth performance during the entire growth period from weaning to market. In total, 96 weaned barrows were group-housed in a BSL-2 containment facility and allotted to 1 of 3 experimental treatments that were maintained throughout the study: non-infected pigs received an ISF-devoid control diet (**NEG**, *n*=24), and infected pigs received either the control diet (**POS**, *n*=36) or that supplemented with total ISF in excess of 1,600 mg/kg (**ISF**, *n*=36). Following a 7-day adaptation, weanling pigs were inoculated intranasally with either a sham-control (**PBS**) or live PRRSV (1×10^5 $TCID_{50}$/mL, strain NADC20). After inoculation, individual blood samples (*n*=8-12/treatment) were routinely collected to monitor viral clearance and hematological parameters, including serum neutralizing anti-PRRSV antibody production. Pen-based oral fluids were used to monitor PRRSV clearance at later growth stages. A 1- or 2-way ANOVA was performed to compare experimental treatments depending on whether the outcome was repeatedly measured. In general, PRRSV infection decreased performance during early growth phases, resulting in 5.4% lower final BW

[6]Associated Manuscript: Smith, B. N., M. L. Oelschlager, M. S. Abdul Rasheed, and R. N. Dilger. 2020. Dietary soy isoflavones reduce pathogen-related mortality in growing pigs under porcine reproductive and respiratory syndrome viral challenge. J. Anim. Sci. 98(2): 1-15. doi: 10.1093/jas/skaa024.

for POS vs. NEG pigs ($P < 0.05$). Dietary ISF elicited inconsistent effects on growth performance, increased ($P < 0.05$) neutrophil cell counts and the relative proportion of memory T-cells, and decreased ($P < 0.05$) the time to full PRRSV clearance from oral fluids. Dietary ISF also elicited a more robust anti-PRRSV neutralizing antibody production as compared with POS pigs. Additionally, and most notably, POS pigs experienced ~50% greater infection-related mortality rate vs. ISF pigs ($P < 0.05$), which may have significant economic implications for producers. Overall, dietary ISF ingestion supported immune responses and reduced mortality in PRRSV-infected pigs when fed to growing pigs though the biological mechanism of these effects remains unclear.

Introduction

Porcine reproductive and respiratory syndrome virus (**PRRSV**) continues to be one of the leading pathogens causing high economic impact in modern swine production (Holtkamp et al., 2013). Due to its prevalence and lack of highly effective vaccine options (Loving et al., 2015), nutritional intervention or management strategies are of high interest for PRRSV mitigation due to relatively low cost and ease of implementation. It has been demonstrated that increasing the level of soybean meal in diets fed to pigs under immune stress reduces adverse effects of illness and may improve growth performance (Boyd et al., 2010; Rochell et al., 2015). However, those studies were unable to determine if improved growth performance was due to differences in dietary bioavailable amino acid content or the presence of other dietary components. Isoflavones (**ISF**) are flavonoid compounds that are enriched in soybeans and possess anti-inflammatory and anti-oxidative properties (Smith and Dilger, 2018). More specifically, under PRRSV-challenge, dietary ISF have been shown to beneficially alter adaptive immune responses in weaned pigs by improving cytotoxic-to-helper T-cell ratios, which may elicit performance benefits during the

recovery period (Smith et al., 2019). The objective of this experiment was to recreate immunological effects previously observed in young pigs infected with PRRSV receiving ISF and determine how those immunomodulatory effects influence growth performance during the entire growth period from weaning to market. Our hypothesis was that pigs infected with PRRSV receiving ISF would recover from PRRSV-infection faster and thus exhibit better growth performance over the whole growth period.

Materials and Methods

The protocol for this experiment was approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of the University of Illinois at Urbana-Champaign.

Animal Husbandry and Experimental Design

Ninety-six weanling pigs (96 barrows; 6.77 ± 0.94 kg initial body weight, **BW**) were obtained from a PPRS-negative, non-PRRS vaccinated commercial herd (1050 Cambro genetics; Carthage Veterinary Service, Ltd.) and group-housed at the Innovative Swine Solutions Veterinary Research Facility (**ISS-VRF**), a Biosafety Level 2 production containment facility in Champaign, IL. Pigs remained at this facility and allowed to grow to market weight (approximately 27 weeks of age, 118-129 kg average BW) before being transported to the Meat Science Laboratory at the University of Illinois for slaughter. The ISS-VRF consisted of 4 individual production rooms (30' x 14') with 4 pens (up to 6 pigs per pen) available to house pigs in each room. Each room was equipped with a high-efficiency particulate air filtration system, plastic slotted flooring appropriate for market-sized pigs, and each pen provided appropriate floor space for pigs taken to market weight. Lighting and ambient temperature was maintained as appropriate for life stage.

Upon entry to the facility, pigs were weighed and uniformly allotted to 1 of 3 experimental treatments [*n* = 24 for non-PRRSV-infected controls (**NEG**), *n* = 36 each for PRRSV-infected controls (**POS**) and PRRSV-infected receiving soy isoflavones (**ISF**)] by completely randomized design based on initial body weight. All replicates of the NEG treatment (6 pigs per replicate, 4 replicates total) were housed in a single room to account for biosecurity while replicates for POS and ISF treatments (6 pigs per replicate, 6 replicates total) were equally represented in the remaining three rooms at ISS-VRF. Two levels of supplemental ISF (none vs. ≥1,600 mg/kg Novasoy400; ADM, Decatur, IL) constituted the total of two dietary treatments (**Table 4.1**). The ISF provided to the ISF treatment are reflective of those typical for a commercially relevant corn-soybean meal diet fed to pigs with approximately 20% soybean meal inclusion. Both diets contained soy protein concentrate (**SPC**) as a protein source, which is practically devoid of soy isoflavones. Experimental diets were formulated to be isocaloric and, with the exceptions of corn and the isoflavone-enriched product, were identical in ingredient composition. Experimental diets were fed over 7 feeding phases including nursery phases 1 and 2 (**N1**-**N2**) and finisher phases 1-5 (**F1**-**F5**). Isoflavone and saponin concentrations of ingredients and experimental diets were quantified via HPLC at the USDA-ARS National Center for Agricultural Utilization Research (Peoria, IL) according to procedures of Berhow et al. (2006). Crude protein was determined by measuring total nitrogen (TruMac N, Leco Corp., St. Joseph, MI) as standardized with EDTA and amino acid concentrations were determined at the University of Missouri Agricultural Experiment Station (Columbia, MO; **Table 4.2**) according to AOAC (2002) official methods [920.39 and 982.30 E(a, b, c), for crude protein and amino acid concentrations, respectively]. Gross energy of the experimental diets was determined using an adiabatic bomb calorimeter (Parr Instruments, Moline, IL), and DM (method 934.01, AOAC

International, 2002) and OM were performed by determining percent ash (method 942.05, AOAC International, 2002) and subtracting from 100. Diets were analyzed for total dietary fiber according to Prosky et al. (1994), but no separation of soluble and insoluble fractions was made. Diets were formulated on a standardized ileal digestible (**SID**) amino acid basis with identical concentrations across all diets (**Tables 4.2 and 4.3**). All diet formulations met or exceeded NRC (2012) nutrient recommendations for each life stage. It should be noted that after the delivery of finisher diet 3 (**F3**), we became aware that both diets contained less than the recommended amount of phosphorus for the first 5 feeding phases. This error impacted the Ca-to-P ratio, but this deficit was corrected in the finisher 4 (**F4**) and 5 (**F5**) diets. Analyzed nutrient composition for each diet are presented in **Tables 4.4 and 4.5**.

Following a 1-week adaptation period to experimental diets [days post-inoculation (**DPI**) -7 to 0], blood was collected from 2 pigs per pen to establish 0 DPI baseline measurements and to ensure that a representative number of pigs were confirmed PRRSV-negative at experiment initiation. Immediately following blood collection, all pigs were administered via intranasal inoculation either 2 mL of a 2% fetal bovine serum + phosphate-buffered saline solution (sham-control) or 1.0×10^5 50% tissue culture infective dose of PRRS virus (strain NADC20, courtesy of Dr. Federico Zuckermann, University of Illinois, Urbana, IL).

Growth Performance

Growth performance was recorded on a pen basis. Individual pig and pen feeder weights were captured weekly from -7 to 48 DPI, every other week from 48 to 118 DPI, and every third week from 118 to 161 DPI to accommodate increased heat stress associated with late summer weather during the end of the experiment for calculation of average daily gain (**ADG**), average daily feed intake (**ADFI**), and feed efficiency (gain-to-feed, **G:F**). Growth performance data are

reported in reference to our inoculation schedule (e.g., -7 to 0 DPI). Mortality was also recorded throughout the experiment and growth performance measurements were adjusted to reflect mortalities within individual replicate pens.

Rectal Temperatures

Individual pig served as the experimental unit for rectal temperatures. Rectal temperatures were recorded using a commercial-grade livestock probe thermometer (GLA M700 Thermometer, GLA Agricultural Electronics, San Luis Obispo, CA) and collected from up to 3 pigs per pen (48 pigs total, same pigs collected when possible) at 0, 3, 6, 10, and 13 DPI. If blood collection was occurring on the same day as rectal temperature collection, the rectal temperature was captured prior to bleeding.

Whole Blood and Sera Collection and Measurements

Individual pig served as the experimental unit for all whole blood and serum outcomes. Blood samples (up to 8 mL total) were collected from the jugular vein of up to 2 pigs per pen into Vacutainer blood collection tubes (BD, Franklin Lakes, NJ) at 0, 3, 6, 13, 20, 27, and 34 DPI using a 21 gauge needle. Whole blood samples collected on 0, 3, 6, and 13 DPI were collected into tubes containing EDTA as an anti-coagulant, placed on ice, and submitted to the University of Illinois College of Veterinary Medicine Clinical Pathology Lab for complete blood cell counts and differential analyses using a multi-parameter, automated hematology analyzer (CELL-DYN 3700, Abbott Laboratories, Abbott Park, IL). Additional whole blood was collected on 13 and 20 DPI for a T-cell immunophenotyping protocol performed in-house via flow cytometry (protocol described below). Serum samples collected on 0, 3, 6, and 13 DPI were collected into serum tubes, allowed to clot at room temperature, and centrifuged at 1,300 × *g* for 15 min at 20ºC before storage in 0.5 mL aliquots at -80ºC pending subsequent analyses. One aliquot per pig was

submitted to the Veterinary Diagnostic Laboratory for detection of PRRS viral antigen via quantitative real-time polymerase chain reaction (**qRT-PCR**) using Z-PRRSV Multiplex reagents (Tetracore, Rockville, MD) as described in Smith et al. 2019. Additional serum aliquots were separated and saved for the following assays: 1) submission to the Clinical Pathology Lab for a general health serum chemistry panel measured at 0, 3, 6, and 13 DPI and 2) submission to the University of South Dakota Animal Disease Research and Diagnostic Laboratory for detection of PRRSV-neutralizing antibodies measured at 20, 27, and 34 DPI.

T-Cell Immunophenotyping

Whole blood collected on 13 and 20 DPI was used for isolation of peripheral blood mononuclear cells (**PBMC**) for a T-cell immunophenotyping procedure using flow cytometry. In brief, PBMC were isolated by placing individual whole blood samples over a density gradient [SepMate 15 (IVD) and Lymphoprep, StemCell Technologies, Cambridge, MA] and centrifuging at 1,200 × *g* for 25 minutes at 20°C. Isolated PBMC were then washed with phosphate buffer saline containing 2% fetal bovine serum before being counted using the Moxi Z Mini Automated Cell Counter (ORFLO Technologies, Ketchum, ID). Individual samples were prepared with 1.0×10^6 cells and labeled with external fluorescent antibodies against cell surface markers CD3 (FITC Mouse Anti-Pig CD3ε, BD Pharmingen, San Jose, CA), CD4 (Alexa Fluor 647 Mouse Anti-Pig CD4a, BD Pharmingen, San Jose, CA), and CD8 (PE Mouse Anti-Pig CD8b (BD Pharmingen, San Jose, CA). Following application of external antibodies for 45 minutes at 4°C, cells were fixed using 4% paraformaldehyde solution for 20 minutes at 4°C. Prepared, labeled cells were evaluated at the University of Illinois Flow Cytometry Facility using a BD LSR II Flow Cytometry Analyzer (BD Biosciences, San Jose, CA) and flow cytometer outputs were analyzed using FCS Express 5 Plus (De Novo Software, Glendale, CA).

To summarize our analysis and gating procedures, recorded cellular events were plotted by density using forward and side scatter areas on the y- and x-axes, respectively, to exclude dead cells and cellular debris and allow for the selection of lymphocyte cells based on size. A single gate was applied to the lymphocyte population, the content of which was then plotted by density using forward scatter width and side scatter on the y- and x-axes, respectively, to allow for the gating and analysis of single cell events within our lymphocyte population only. Utilizing single cellular events within our lymphocyte population, single-stain control samples were used to set our detection thresholds for each individual fluorochrome and creation of a CD3+ gate. Gated on CD3+ lymphocytes, a final density plot using our CD8+ and CD4+ fluorochrome channels on the y- and x-axes, respectively, was created and our individual fluorochrome detection levels were used to apply quadrants to the density plot. Statistics from each quadrant were then used for our individual effector T-cell proportions (e.g., Helper T-cells, CD3+/CD4+).

Oral Fluid Analyses

Oral fluids were collected weekly on a pen basis starting at 39 DPI through 123 DPI or until a single pen had 2 consecutive negative test results in a row. Ropes were hung at the front of each pen in two locations and pigs were allowed to chew on the ropes for 15 to 20 minutes or until the ropes were saturated with salvia. Saliva was manually expressed from the ropes into a clean plastic bag and transferred to 15 mL conical tubes for submission to the University of Illinois College of Veterinary Medicine Veterinary Diagnostic Laboratory for detection of PRRS viral antigen via qRT-PCR. Samples that could not be submitted the same day were stored temporarily at -20°C pending analysis.

Statistical Analyses

Statistical analysis was dependent on whether outcomes were measured at a single time-point or at multiple time-points for the same subject. For calculations of ADG, ADFI, G:F, and oral fluid samples, individual pens holding up to 6 pigs served as the experimental unit. For body weight, all immune and clinical outcomes, and mortality, individual pig was considered the experimental unit, with 12 to 18 individual pigs utilized for each of the three experimental treatments for immune and clinical outcomes. A total of two different diets were fed to PRRSV-infected pigs, while a single group of uninfected pigs received only the control diet. Thus, the 1- or 2-way analyses of variance (ANOVA) described below were performed to evaluate effects between diets treatments.

For all single time-point outcomes, a 1-way ANOVA was conducted using the MIXED procedure of SAS Enterprise Guide (version 6.1) (SAS Institute, Inc., Cary, NC). For repeated measures, a 2-way ANOVA including a time effect was conducted for all outcomes involving samples collected from the same subject at multiple time-points. When appropriate, means separation was conducted. Least-square means and the standard error of the mean estimates were derived from the 1- and 2-way ANOVA. In all cases, outliers were identified as having an absolute Studentized residual value of 3 or greater and statistical significance was considered at $P \leq 0.05$.

Results

Growth Performance

Results for growth performance can be found in **Figure 4.1 and Table 4.6**. Between NEG and POS treatments, PRRSV-infection resulted in decreased individual body weight starting at 13 DPI ($P < 0.05$) through to the end of the live-phase of the experiment (161 DPI).

Regarding growth performance metrics, PRRSV-infection decreased ADG during growth periods 0 to 6 DPI, 6 to 13 DPI, and 13 to 20 DPI ($P < 0.05$) and decreased ADFI during growth periods 6 to 13 DPI and 13 to 20 DPI ($P < 0.05$). However, PRRSV-infected pigs relative to non-infected controls only exhibited reductions in feed efficiency during the 0 to 6 DPI growth period ($P < 0.05$) and again much later during the 104 to 118 DPI growth period ($P < 0.05$).

Between POS and ISF treatments, there were differences observed for individual body weights ($P < 0.0001$). Ingestion of supplemental ISF resulted in decreased body weights during the later portion of the growth period (104, 118 and 161 DPI) ($P < 0.001$), but no differences were observed during the acute infection or early recovery phases and the difference at 161 DPI was not maintained when pigs were weighed a few days later prior to slaughter at the University of Illinois Meat Science Laboratory. Regarding growth performance metrics, ISF supplementation increased ADFI during the 0 to 6 DPI growth period ($P < 0.05$) and the 90 to 104 DPI growth periods, but decreased ADFI during the 76 to 90 DPI ($P < 0.05$). Moreover, ISF supplementation decreased growth efficiency, as measured by G:F, during the 90 to 104 DPI growth period ($P < 0.05$), but increased growth efficiency during the 104 to 118 DPI growth period ($P < 0.05$). Across the entire growth period, however, all effects of ISF supplementation on growth performance were transient and inconsistent with the exception of individual body weight.

Rectal Temperatures

Results for rectal temperature can be found in **Table 4.7**. Between NEG and POS treatments, PRRSV-infection increased rectal temperatures significantly by 6 DPI ($P < 0.05$) with rectal temperatures of infected pigs returning to the normal range by 13 DPI, indicating successful establishment of infection. Between POS and ISF treatments, there were observed

differences in rectal temperatures ($P < 0.05$), with ISF supplementation causing increased rectal temperatures at 0 DPI and decreased rectal temperatures at 6 DPI ($P < 0.05$).

Complete and Differential Blood Cell Counts

For the sake of clarity, results for the erythrogram and leukogram analyses are presented and discussed separately.

Erythrogram Results

Compared with the NEG treatment, PRRSV-infection resulted in decreased red blood cell (**RBC**) counts, decreased hemoglobin concentrations, and decreased hematocrit on 6 and 13 DPI (**Figure 4.2**) and decreased mean cellular hemoglobin concentrations (**MCHC**) on 6 DPI ($P < 0.05$). These findings are indicative of an active infection, with many immune challenges resulting in decreased RBC synthesis and release into circulation. Between POS and ISF treatments, ISF supplementation resulted in decreased RBC counts and hematocrit at 13 DPI ($P < 0.05$). Full panel results for the erythrogram analysis can be found in **Table 4.8**.

Leukogram Results

Between NEG and POS treatments, PRRSV-infection resulted in decreased white blood cell (**WBC**) counts on 3, 6, and 13 DPI, increased proportions of circulating neutrophils at 6 and 13 DPI, and decreased proportions of circulating lymphocytes at 6 and 13 DPI ($P < 0.05$) (**Figure 4.3**), which is in agreement with our immunophenotyping data (see below). When looking at the total counts for neutrophils, however, we observed that the POS and ISF treatments actually had fewer circulating neutrophils at 3 DPI compared with non-infected controls ($P < 0.05$), which was maintained for the POS treatment at 6 DPI. These numbers increased for our POS treatment by 13 DPI, but supplemental ISF resulted in greater numbers of circulating neutrophils to levels similar to or greater than that of our negative controls at 6 and 13

DPI. Total lymphocyte counts were consistent with the calculated proportions observed. The neutrophilia was unexpected for a viral infection model, however, due to the high incidence of secondary bacterial infections observed for this experiment it could be considered a reasonable finding. More interestingly, the difference in total neutrophil counts across the acute infection period could be worth noting in the context of these bacterial insults following PRRSV-infection. Full panel results for the leukogram analysis can be found in **Table 4.9.**

Serum Viral Load, Anti-PRRSV Antibody Titers, and Neutralizing Anti-PRRSV Antibody Titers

Results for serum PRRS viral RNA concentrations can be found in **Table 4.10**. Between NEG and POS treatments, PRRSV-infection resulted in viremia detectable by serum PRRS viral RNA by qRT-PCR at 3, 6, and 13 DPI. Non-infected control pigs remained PRRSV-negative for serum PRRS viral RNA throughout the course of the experiment. Between POS and ISF treatments, there was no difference observed for serum PRRS viral RNA concentrations at any time-point measured ($P > 0.05$).

Results for PRRSV neutralizing antibody titers can be found in **Figure 4.4**. Across all time-points measured, the NEG treatment tested negative for PRRSV neutralizing antibodies (i.e., titer of <1:4). At 20 DPI, 8 out of 11 POS pigs collected had negative titers, 2 had titers of 1:4, and 1 had a titer of 1:8. Within the ISF treatment, 8 out of 12 pigs collected had negative titers and 4 had titers of 1:4. At 27 DPI, 3 of the 12 POS pigs collected had negative titers, 1 had a titer of 1:4, 4 had titers of 1:8, 1 had a titer of 1:16, 2 had titers of 1:32, and 1 had a titer of 1:128. Within the ISF treatment, none of the 12 pigs collected tested negative, 5 had titers of 1:4, 4 had titers of 1:8, 1 had a titer of 1:16, and 2 had titers of 1:32. At 34 DPI, none of the 11 POS pigs collected tested negative, 1 pig had a titer of 1:4, 2 had titers of 1:8, 4 had titers of 1:16, 3

had titers of 1:32, and 1 had a titer of 1:64. Within the ISF treatment, none of the 12 pigs collected tested negative, 2 had titers of 1:8, 6 had titers of 1:16, and 4 had titers of 1:32. When looking specifically at pigs that had clinically protective titers (i.e., ≥ 1:8), 9% (1/11) of pigs collected in the POS treatment had protective titers at 20 DPI vs. 0% (0/12) in the ISF treatment. At 27 DPI, 72% (8/11) of pigs collected in the POS treatment had protective titers vs. 58% (7/12) in the ISF treatment. At 34 DPI, 83% (10/12) in the POS treatment had protective titers vs. 100% (12/12) in the ISF treatment.

PRRSV Oral Fluid Clearance

Results for PRRSV clearance in oral fluids by treatment can be found in **Figure 4.5.** Monitoring of PRRSV clearance in oral fluids on a pen basis began on 39 DPI and was continued on a weekly basis through 123 DPI. The NEG treatment remained negative for oral PRRSV antigen by qRT-PCR throughout the course of the experiment. The POS treatment peaked with 100% of pens being oral-fluid-positive at 60 DPI and experienced an initial wave of clearance at 74 DPI. The ISF treatment peaked later at 67 DPI and experienced an initial wave of clearance at 81 DPI. Both POS and ISF treatments had replicate pens that reentered an infectious stage and cycled between 0 to 33% positive until 102 DPI, with a greater number of ISF replicate pens maintaining their negative status compared with the POS treatment. The ISF treatment reached final clearance at 102 DPI, while the POS treatment did not reach final clearance until 123 DPI.

Secondary Bacterial Infections and Mortality Prevalence

Some level of infection-related mortality can be expected among young pigs infected with PRRSV. However, starting at approximately 10 to 12 DPI, there was an observable increase in infection-related mortalities observed in our POS and ISF treatment groups, above expected

values for this model. Based on clinical presentation and rapid onset of symptoms, the veterinarian overseeing ISS-VRF suspected that our PRRSV-infected pigs were suffering from secondary bacterial co-infection (likely *Streptococcus suis* [***Strep. suis***], though a differential diagnosis of *Haemophilis parasuis* was also suggested) (Gottschalk, 2020; Segales, 2020). Due to the large number of infected pigs impacted by this secondary infection and the increasing number of mortality cases, researchers opted to treat all pigs on study (including uninfected pigs in the NEG treatment) with a three-day course of enrofloxacin (Baytril 100, Bayer Healthcare LLC, Shawnee Mission, KS) starting at 21 DPI in attempt to slow infection-related losses.

Results for mortality rate and extent by treatment can be found in **Figure 4.6**. Of the original 96 pigs allotted, a total of 27 (28%) died prior to the completion of the experiment. Of those 27, 3 were categorized as non-infection/pathogen related deaths: one NEG pig that tested as a false positive for PRRSV serum antigen at 6 DPI, one NEG pig that developed severe kyphosis, and one ISF pig that developed an inguinal hernia. With those excluded, the POS treatment experienced 47% mortality while the ISF treatment experienced only 25% mortality. The initial rate of mortality appeared similar between POS and ISF treatments between 7 and 21 DPI, but the rate slowed for the ISF treatment beyond that point. Recall that all pigs were treated with antibiotics at 21 DPI in attempt to control increased losses attributed to a severe secondary infection with *Strep. suis*. The POS treatment continued to experience infection-related mortalities as late as 77 DPI, while the ISF treatment experienced their last infection-related mortality at 44 DPI.

T-Cell Immunophenotyping Results

Results for T-cell immunophenotyping can be found in **Table 4.11**. Between NEG and POS treatments, PRRSV-infection caused a transient leukopenia (i.e., decrease in total

lymphocytes), with increased proportions of circulating mature T-cells (CD3+), decreased proportions of cytotoxic T-cells (CD3+/CD8+), and decreased proportions of memory T-cells (CD3+/CD4+/CD8+) ($P < 0.05$) on 13 DPI. Proportions of all impacted T-cell immunophenotype parameters in POS animals returned to values comparable to uninfected controls by 20 DPI. Interestingly, ISF increased the relative proportion of memory T-cells over the NEG treatment at 20 DPI. Regarding the helper:cytotoxic T-cell ratio (a measure of immune system functionality), PRRSV-infected treatments exhibited an increased ratio, which is typically a predictor of an appropriate immune response, with the ISF treatment having the greatest ratio value ($P < 0.05$).

Serum Chemistry Results

Results for serum chemistry clinical analyses can be found in **Table 4.12**. The majority of differences seen in analyte concentrations across the acute infection period (0 to 13 DPI) appear to be due to PRRSV-infection alone with differences mainly observed during the 6 and 13 DPI time-points ($P < 0.05$). Regarding measures of protein status, POS and ISF treatments exhibited increased creatine concentrations, decreased creatine phosphokinase (**CK**) activity, and decreased total blood proteins (but increased globulin proteins at 13 DPI), which together can indicate disruptions in protein metabolism and skeletal muscle deposition, with the exception of globulin proteins which mainly indicate an active immune response. Regarding serum mineral and electrolyte concentrations, POS and ISF treatments exhibited decreased calcium, potassium, and sodium concentrations and increased chloride concentrations across the acute infection period compared with the NEG treatment. PRRSV-infection also reduced blood glucose levels fairly dramatically across the acute infection period, which is expected due to the increase demand for glucose during infection and decreased feed intake. Finally, PRRSV-infection

resulted in several changes observed for liver enzyme serum concentrations (i.e., decreased alkaline phosphatase concentrations, decreased glutamate dehydrogenase concentrations, increased aminotransferase concentrations) across infected treatments.

There were only a few analytes at specific time-points during the acute infection period that were influenced by dietary ISF. At 6 DPI, pigs in the ISF treatment group had lower blood glucose levels than the NEG and POS treatments, but did not differ from the POS treatment at any other time-point measured ($P < 0.05$). At 13 DPI, pigs in the ISF treatment group had similar sodium concentrations to those in the NEG treatment ($P < 0.05$). Additionally, at 13 DPI pigs in the ISF treatment had greater ($P < 0.05$) total CO_2 serum concentrations than the NEG treatment, with the POS treatment exhibiting intermediate concentrations.

Discussion

Isoflavones are bioactive components that are found in high concentrations in soybeans and soybean-derived feedstuffs and have been shown to have antiviral activity (Wang and Murphy, 1996). This activity has been demonstrated against PRRSV specifically in live animal studies previously (Greiner et al., 2001a; Greiner et al., 2001b; Rochell et al. 2015; Smith et al., 2019). The purpose of this study was to help elucidate a time-dependent biological mechanism by which ISF exert immunomodulatory effects over the entire infection period with PRRSV, both acute and recovery phases, which was not fully captured by these previous experiments.

Induction of PRRSV infection had the greatest impact on growth performance. PRRSV-infected pigs had lower body weights as early at 13 DPI and final finishing weights approximately 7% lighter than that of non-infected controls. Isoflavones only had transient impacts on growth performance with no consistent trend over the growth period. Previously, the isolated isoflavones genistein and daidzein were independently found to improve ADG during

the early post-inoculation period (Greiner et al., 2001a; Greiner et al., 2001b), but those same effects have not been consistently observed in situations where a mixture of isoflavones to mimic naturally occurring soy ISF ratios is fed. In PRRSV-challenged pigs, those receiving higher concentrations of SBM did not experience a decrease in average daily gain, a hallmark of PRRSV infection, however researchers could not conclude if that improvement was due to increased crude protein in the diet or bioactive compounds such as isoflavones (Rochell et al., 2015). However, in another PRRSV-challenge study evaluating the effects of dietary SBM on amino acid digestibility and losses, researchers found that a 3-time increase in dietary SBM, which would have significantly increased the ISF content in experimental diets, did not affect growth performance in infected pigs (Schweer et al., 2018). Most recently in our laboratory, PRRSV-infected weaned pigs infected with the same strain of PRRSV virus utilized for this study exhibited greater feed efficiency when receiving supplemental ISF between 6 to 14 DPI, but ISF did not impact any other growth metrics over the 2-week infection period (Smith et al., 2019). It should be noted that the PRRSV strains used in some those studies are different than what was utilized in our study. In that context, findings from the current study and previous research suggest that soy isoflavones may have some influence on growth performance under PRRSV-challenge, but that influence is not consistent over the entire infection period. Additionally, differences in virulence of individual PRRSV strains and the severity of clinical disease they cause (Johnson et al., 2004) may be a factor in the capacity of which isoflavones are able to influence growth performance in pathogenically-stressed pigs and partially explain differences observed between PRRSV-challenge studies. Regardless, while there does not seem to be a clear explanation for why the final on-site live body weights for our ISF treatment were approximately 3% lower compared with the POS treatment, this appears to be a transient effect

since no differences were detected between the two dietary treatments at time of slaughter later the same week.

Shifting the focus from growth performance to immune response and recovery outcomes, regarding rectal temperatures as an indicator of ongoing infection, pigs in the ISF treatment had increased rectal temperatures at 0 DPI but decreased rectal temperatures at 6 DPI compared with the POS treatment. Reasoning for the increased baseline, pre-inoculation rectal temperatures of ISF supplemented pigs is unclear, however, due to fact that both the POS and ISF treatments are within the normal physiological rectal temperature range for pigs of that age (38.6°C-39.5°C), the biological relevance of that difference may be considered weak. The half-degree difference between POS and ISF treatments at 6 DPI, however, is physiologically relevant, with ISF supplementation reducing rectal temperatures of infected pigs to temperatures similar to those observed in the negative control animals.

Regarding erythrocyte measures, starting at 6 DPI we observed a decline in total RBC counts and hematocrit in both infected treatments, which has been observed previously in PRRSV-challenge models (Halbur et al., 2002). By 13 DPI, the ISF treatment had the lowest RBC counts and hematocrit values, with the POS treatment exhibiting intermediate levels between the ISF and negative control treatment. While within normal clinical ranges, the levels of the ISF treatment group were nearing or exceeding lower cutoff values for those measures (Friendship & Henry, 1992). It is thought that a potential cause of anemia during infection may be mediated by systemic pro-inflammatory cytokines, which can induce erythroid hypoplasia due to increased demand for granulocyte production from myeloid progenitor cells (Halbur et al., 2002). If true, then one explanation for why ISF consumption elicited a more severe anemia could be that pigs receiving ISF experienced a heavily neutrophilic response. Such a case has

been observed with higher levels of systemic inflammation, earlier during the infection period, thus creating a stronger suppression on erythrocyte differentiation (Duncan et al., 1994). This is particularly interesting in the context of our PRRSV-challenge since PRRSV is considered to be immunosuppressive, especially during the early acute infection period, and the fact that pigs on study suffered severe secondary bacterial infections.

Upon initial exposure to a viral insult, tissue-resident innate immune cells such as macrophages and dendritic cells typically produce the anti-viral, pro-inflammatory type I interferons, interferon-α and interferon-β (**IFN-$\boldsymbol{\alpha}$**, **IFN-$\boldsymbol{\beta}$**) (Loving et al., 2015; Bogdan, 2000). Targeting pulmonary alveolar macrophages specifically, PRRSV dampens this type-I interferon response, with peak IFN-α titers being minimally 1,000-fold lower than that observed during other common swine respiratory viruses (e.g. swine influenza virus, **SIV**, and porcine respiratory circovirus, **PRCV**). It also appears to reduce the production of other key pro-inflammatory cytokines such as TNF-α (van Reeth et al., 1999). Suppression of these signaling pathways results in poor viral clearance and a transient leukopenia and lymphopenia (Shimizu et al., 1996; Albina et al., 1998; Bogdan, 2000). We observed this in PRRSV-infected pigs, where decreased WBC and lymphocyte counts were observed starting as early as 3 DPI. However, suppression of type-I interferon signaling and recruitment of adaptive immune cells does not explain differences in neutrophil counts between our two infected treatments, as granulocytes are not typically associated with PRRSV infection. However, previous PRRSV models have observed transient increases in total granulocyte percentages during early infection which is in line with our findings (Hernandez et al., 2018). Regardless, some of this response may be explained by the estrogenic activity of ISF and the role of estrogen receptors on immune cells, which may have

impacted the susceptibility of our ISF treatment to secondary bacterial infections (Smith and Dilger, 2018; Straub, 2007; Yakimchuk et al., 2013).

Due to their structural similarity to 17β-estradiol (**E2**), isoflavones have the capacity to interact with estrogen receptors throughout the body as a weak E2 analog. There are two main types of estrogen receptors, estrogen receptor-α (**ER-$\boldsymbol{\alpha}$**) and estrogen receptor-β (**ER-$\boldsymbol{\beta}$**); of the two, soy ISF preferentially bind ER-β (Andres et al., 2009). Additionally, while more typically thought to express anti-inflammatory effects, certain cell responses mediated by ER-β are more appropriately categorized as pro-inflammatory. ER-β can be found throughout the body, but is notably found on peripheral leukocytes, tissue macrophages, and pulmonary epithelial tissues. One pro-inflammatory response by ER-β activity is increased apoptotic activity. In response to local inflammation or pathogenic insult, the expression of ER-β increases on immune cells which in turn increases pro-apoptotic pathway activation (Straub, 2007; Yakimchuk et al., 2013). It is possible that the presence of circulating ISF in pigs fed ISF may have resulted in increased apoptosis of PRRSV-infected alveolar macrophages, potentially preventing spread of PRRSV to uninfected alveolar macrophages and affording them greater functionality during a secondary bacterial insult at the level of the lung. Since it is likely that the pigs utilized in the experiment were exposed and asymptomatically harboring pathogenic bacteria prior to their arrival and inoculation with PRRSV, dietary ISF may have afforded ISF pigs increased protection against bacterial insult in the face of a PRRSV infection, allowing local immune cells to recruit an appropriate neutrophil response earlier than pigs in the POS treatment. Additionally, human studies suggest that increased levels of E2 increases total numbers of neutrophils in circulation and may contribute to greater phagocytic activity in those cells; being an E2 analog, ISF may be directly contributing to the rise in neutrophil counts of our ISF treatment (Bouman et al., 2005).

We provide this purported mechanism but were unable to collect lung tissue from infected pigs during acute infection to assess local cytokine levels or expression or cellular damage, so we cannot confirm whether secondary bacterial infections started in the respiratory tract. We can confirm that systemic serum cytokine concentrations did not differ between infected treatments (results not shown).

Within the broad clinical picture, the impact of ISF on immune cell numbers and mobilization may be what set the stage for recovery and greater survival by our ISF treatment. By having some influence on the kinetics of the immune response to a secondary bacterial infection, pigs receiving ISF were able to begin the recovery process from PRRSV more completely than our POS treatment. This was reflected in a few of our immune measures captured. First, our ISF treatment elicited a greater proportion of memory T-cells by 20 DPI, which are typically a group of adaptive immune cells that are relatively inactive in a PRRSV infection, but are recognized as important cells for the development of immunological memory (Lanzavecchia and Sallusto, 2005; Fu et al., 2012). Additionally, though it is in contrast to previous findings by our lab (Smith et al., 2019) and not necessarily characteristic a PRRSV infection, we found PRRSV-infected treatments exhibited an increased helper:cytotoxic T-cell ratios, with the ISF treatment having the greatest ratio value across all treatments. However, immune responses to secondary bacterial infections may have played a role in influencing this outcome. Second, while it did not seem to increase the rate of neutralizing antibody production, more pigs in the ISF treatment demonstrated protective titers by our last collection point (34 DPI) compared with the POS treatment, which was approximately 10 days following antibiotic administration. Though sampled from a small number of animals, these results suggest that ISF promote greater immune protection across an infected population, which is a key factor for

PRRSV clearance and recovery. Third, and interestingly, while a majority of replicate pens in the POS treatment started to test negative by oral fluids two weeks before the ISF treatment, half of the POS replicates continued to cycle back into positive status 5 weeks beyond when a majority of ISF replicate pens tested oral fluid negative for the first time. These findings together suggest that once the ISF treatment started to enter the recovery phase from PRRSV, they did so more completely and were able to reach true clearance 7 weeks earlier (with the exception of one replicate pen) than the POS treatment.

While reaching PRRSV clearance earlier in the growth period is of obvious interest to swine producers, what ISF really afforded the PRRSV-challenge pigs within this experiment was fewer infection-related losses. The ISF treatment experienced approximately 25% mortality over the entire growth period compared with the POS treatment with nearly 50% mortality. These mortality patterns suggest that under PRRSV-challenge with a severe secondary bacterial infection that ISF may contribute to reduced overall mortality rates of infected pigs. As a side note, we concluded that based on our mortality patterns it appears that the incorrectly formulated Calcium: Phosphorus ratio in the initial finisher phase diets did not contribute strongly to overall mortality as all experimental treatments were affected and we observed an appropriate level of mortality in the uninfected, control-fed group throughout the experiment. Even in the absence of any calculated production losses, we conclude that this finding may have significant financial implications for pork productions when nearly twice as many pigs were able to make it through their entire growth period when they received ISF compared with those that did not when faced with a severe pathogenic challenge.

Unfortunately, it is still not clear exactly how ISF worked biologically within this model. While we have suggested that it may be possible that ISF acted via estrogen-signaling pathways

to impact early immune responses that later impacted recovery from PRRSV complicated with secondary bacterial infections, it is important to note that ISF are a weak estrogen analog, so their activity against pathogens is likely not through those pathways exclusively (Andres et al., 2009; Smith and Dilger, 2018). Additionally, we must keep in mind that this was a nutritional intervention and ISF are heavily metabolized by both host and microbial enzymes in the gastrointestinal tract. More research is warranted to elucidate whether these effects were due to direct activity by circulating isoflavones or if shifts in microbial populations could also be contributing. Finally, these findings were observed under severe pathogen challenge when an isoflavone mixture was added back to the diet at levels normally found in traditional commercial swine diets. Moving forward, there is there is also a need to identify whether higher isoflavone concentrations or a less severe pathogenic challenges would generate more or similar protection against infection-related losses when pigs are faced with PRRSV in a commercial setting.

Implications

Under PRRSV-challenge complicated by severe secondary bacterial infections, dietary soy isoflavones reduced pathogen-associated mortality by approximately 50% over the course of the entire growth period from weaning to market. While having minimal to no impact on growth performance, isoflavones appeared to alter the kinetics of early acute immune responses, impacting the timeline for PRRSV recovery and clearance. This has significant implications for pork producers, however more research is required to further clarify the mechanism of action for these findings and if isoflavone-enriched diets would be beneficial in modern pork production systems.

Tables and Figures

Table 4.1. Experimental treatments[1]

Treatment	Dietary Treatment	Infection Status
NEG	Control diet	Uninfected
POS	Control diet	PRRSV-Infected
ISF	Control diet + ISF[2]	PRRSV-Infected

[1]Abbreviations: PRRSV, porcine reproductive and respiratory syndrome virus; ISF, isoflavones.
[2]ISF provided by Novasoy 400 (ADM, Decatur, IL) at >1,600 mg/kg complete diet across all feeding phases.

Table 4.2. Ingredient and calculated composition of experimental diets (as-fed basis), nursery phases[1]

	Nursery Phase 1		Nursery Phase 2	
Item	**CON**	**CON+ISF**	**CON**	**CON+ISF**
Ingredients, g/kg				
Corn	629.05	624.55	606.20	601.70
Soy protein concentrate[2]	175.00	175.00	200.00	200.00
Dried whey	120.00	120.00	--	--
DDGS	--	--	150.00	150.00
Fish meal[3]	40.00	40.00	--	--
Fat, choice white grease	10.00	10.00	10.00	10.00
Limestone	12.00	12.00	18.00	18.00
Salt	5.00	5.00	6.50	6.50
Vitamin-mineral premix[4]	1.00	1.00	2.25	2.25
Choline chloride, 60%	0.70	0.70	0.70	0.70
Isoflavone mixture[5]	0.00	4.50	0.00	4.50
L-Lys HCl	4.50	4.50	4.25	4.25
DL-Met	1.25	1.25	0.85	0.85
L-Thr	1.00	1.00	0.75	0.75
L-Trp	0.50	0.50	0.50	0.50
Calculated Composition[6]				
ME, kcal/kg	3,470	3,455	3,449	3,434
Crude protein, g/kg	212.4	212.0	227.6	227.2
SID AA, g/kg				
Lysine	14.2	14.2	13.5	13.5
Methionine + Cysteine	7.4	7.4	11.4	11.4
Tryptophan	2.5	2.5	2.4	2.4
Threonine	8.0	8.0	7.4	7.4
Valine	8.9	8.9	9.1	9.0
Isoflavones, mg/kg				
Genistein	2.0	752	2.0	753
Daidzein	1.0	789	1.0	789
Glycitein	0.0	95	0.0	95
Total isoflavones	3.0	1,636	3.0	1,637
Total saponins, mg/kg	664	1,045	759	1,140

[1]All pigs received allotted treatment diet upon starting -7 DPI. Abbreviations: DPI, days post-inoculation; ISF, isoflavones; SID, standardized ileal digestible, AA, amino acids.
[2]Arcon AF, ADM, Decatur, IL.
[3]Special Select™ Menhaden Fish Meal, Omega Protein, Houston, TX.
[4]Vitamin-mineral premix (JBS United, Sheridin, IN) included the following per kilogram of complete diet: Vitamin A (retinyl acetate), 11,128 IU; Vitamin D3 (cholecalciferol), 2,204 IU; Vitamin E (dl-α tocopheryl acetate), 66 IU; Vitamin K (menadione nicotinamide bisulfite), 1.42 mg; Thiamine (thiamine mononitrate), 0.24 mg; Riboflavin, 6.58 mg; Pyridoxine (pyridoxine hydrochloride), 0.24 mg; Vitamin B12, 0.03 mg; d-Pantothenic acid (d-calcium pantothenate), 23.5 mg; Niacin (nicotinamide and nicotinic acid), 44 mg; Folic acid, 1.58 mg; Biotin, 0.44 mg; Cu (copper sulfate), 10 mg; Fe (iron sulfate), 125 mg; I (potassium iodate), 1.26 mg; Mn (manganese sulfate), 60 mg; Se (sodium selenite), 0.3 mg; and Zn (zinc oxide), 100 mg.
[5]Novasoy400, ADM, Decatur, IL.
[6]Metablizable energy and standardized ileal digestible (SID) amino acid values were calculated using NRC (2012). Analyzed crude protein determined as CP = total N × 6.25.

Table 4.3. Ingredient and calculated composition of experimental diets (as-fed basis), grow-finish phases[1]

	Finisher Phase 1		Finisher Phase 2		Finisher Phase 3		Finisher Phase 4		Finisher Phase 5	
Item	**CON**	**CON +ISF**	**CON**	**CON +ISF**	**CON**	**CON +ISF**	**CON**	**CON +ISF**	**CON**	**CON +ISF**
Ingredients, g/kg										
Corn	535.75	531.25	664.15	659.65	815.40	810.90	826.45	821.95	884.35	879.85
Soy protein concentrate[2]	125.00	125.00	100.00	100.00	100.00	100.00	90.00	90.00	85.00	85.00
DDGS	300.00	300.00	200.00	200.00	50.00	50.00	50.00	50.00	--	--
Fat, choice white grease	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Limestone	15.50	15.50	15.50	15.50	14.00	14.00	9.00	9.00	8.00	8.00
Dicalcium phosphate	--	--	--	--	--	--	8.00	8.00	7.00	7.00
Salt	5.00	5.00	4.00	4.00	4.00	4.00	2.00	2.00	2.00	2.00
Vitamin-mineral premix[3]	2.50	2.50	1.50	1.50	1.00	1.00	0.75	0.75	0.75	0.75
Choline chloride, 60%	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70
Isoflavones[4]	0.00	4.50	0.00	4.50	0.00	4.50	0.00	4.50	0.00	4.50
L-Lys HCl	4.50	4.50	3.25	3.25	3.25	3.25	2.30	2.30	1.50	1.50
DL-Met	0.50	0.50	0.10	0.10	0.25	0.25	--	--	--	--
L-Thr	0.25	0.25	0.50	0.50	1.00	1.00	0.50	0.50	0.50	0.50
L-Trp	0.30	0.30	0.30	0.30	0.40	0.40	0.30	0.30	0.20	0.20
Calculated Composition[4]										
ME, kcal/kg	3,434	3,419	3,425	3,410	3,428	3,412	3,418	3,403	3,419	3,404
Crude protein, g/kg	213.6	213.2	179.0	178.6	150.8	150.5	143.6	143.2	130.5	130.1
SID AA, g/kg										
Lysine	11.6	11.5	9.1	9.1	8.7	8.7	7.4	7.4	6.3	6.3
Methionine + Cysteine	10.1	10.1	8.3	8.3	7.4	7.4	6.8	6.8	6.4	6.3
Tryptophan	1.9	1.9	1.6	1.6	1.6	1.6	1.4	1.4	1.2	1.2
Threonine	6.2	6.2	5.5	5.5	5.3	5.3	4.6	4.6	4.2	4.2
Valine	8.2	8.2	6.9	6.9	5.9	5.9	5.6	5.6	5.2	5.1
Isoflavones, mg/kg										
Genistein	1.0	752	1.0	752	1.0	752	1.0	751	1.0	751
Daidzein	1.0	788	1.0	788	1.0	788	1.0	788	1.0	788
Glycitein	0.0	95	0.0	95	0.0	95	0.0	95	0.0	95
Total isoflavones	2.0	1,635	2.0	1,635	2.0	1,635	2.0	1,635	1.0	1,635
Total saponins, mg/kg	474	855	380	760	380	760	342	722	323	703

[1]All pigs received allotted treatment diet upon starting -7 DPI. Abbreviations: DPI, days post-inoculation; ISF, isoflavones; SID, standardized ileal digestible, AA, amino acids.
[2]Special Select™ Menhaden Fish Meal, Omega Protein, Houston, TX.
[3]Vitamin-mineral premix (JBS United, Sheradin, IN) included the following per kilogram of complete diet: Vitamin A (retinyl acetate), 11,128 IU; Vitamin D3 (cholecalciferol), 2,204 IU; Vitamin E (dl-α tocopheryl acetate), 66 IU; Vitamin K (menadione nicotinamide bisulfite), 1.42 mg; Thiamine (thiamine mononitrate), 0.24 mg; Riboflavin, 6.58 mg; Pyridoxine (pyridoxine hydrochloride), 0.24 mg; Vitamin B12, 0.03 mg; d-Pantothenic acid (d-calcium pantothenate), 23.5 mg; Niacin (nicotinamide and nicotinic acid), 44 mg; Folic acid, 1.58 mg; Biotin, 0.44 mg; Cu (copper sulfate), 10 mg; Fe (iron sulfate), 125 mg; I (potassium iodate), 1.26 mg; Mn (manganese sulfate), 60 mg; Se (sodium selenite), 0.3 mg; and Zn (zinc oxide), 100 mg.
[4]Metablizable energy and standardized ileal digestible (SID) amino acid values were calculated using NRC (2012). Analyzed crude protein determined as CP = total N × 6.25.

Table 4.4. Analyzed composition of experimental diets (as-fed basis), nursery phases[1]

	Nursery Phase 1		Nursery Phase 2	
Item	**CON**	**CON+ISF**	**CON**	**CON+ISF**
Dry matter, g/kg	909.2	911.2	903.2	900.1
Organic matter, g/kg	932.9	936.3	946.2	937.9
Crude protein[2], g/kg	218.3	220.3	240.3	238.2
Total dietary fiber, g/kg	99.9	117.5	156.3	156.4
Isoflavones, mg/kg				
Genistein	37.9	988	87.1	1.129
Daidzein	18.5	661	22.2	785
Glycitein	0.0	49.5	0.0	61
Total isoflavones	56.4	1,699	108	1,975
Total saponins, mg/kg	193	560	442	764
Total AA, g/kg	213.0	214.6	230.4	234.1
Indispensable AA, g/kg				
Arginine	12.8	12.9	9.70	14.4
Histidine	5.40	5.50	6.20	6.30
Isoleucine	10.0	10.1	10.6	10.7
Leucine	18.5	18.7	21.3	21.7
Lysine	15.1	15.5	15.3	15.5
Methionine	4.80	4.70	4.50	4.60
Phenylalanine	10.1	10.3	11.6	11.9
Threonine	9.10	9.30	9.20	9.40
Tryptophan	3.10	3.10	3.20	3.00
Valine	11.0	10.9	11.8	12.0
Dispensable AA, g/kg				
Alanine	11.1	11.2	12.3	12.5
Aspartic acid	21.0	21.0	21.8	22.3
Cysteine	3.30	3.30	3.90	3.80
Glutamic acid	37.2	37.3	40.8	41.5
Glycine	9.70	9.70	9.80	9.90
Proline	12.5	12.5	14.6	14.8
Serine	8.50	8.60	9.30	9.70
Tyrosine	6.70	6.90	7.90	8.00

[1]All pigs received allotted treatment diet upon starting -7 DPI. Abbreviations: AA, amino acids; DPI, days post-inoculation; ISF, isoflavones, AA, amino acids.
[2]Analyzed CP = total N × 6.25.

Table 4.5. Analyzed composition of experimental diets (as-fed basis), grow-finish phases[1]

	Finisher Phase 1		Finisher Phase 2		Finisher Phase 3		Finisher Phase 4		Finisher Phase 5	
	CON[2]	CON +ISF	CON	CON +ISF	CON	CON +ISF	CON	CON +ISF	CON	CON +ISF
Dry matter, g/kg	--	888.4	881.0	879.2	864.0	840.4	867.8	867.0	874.5	866.6
Organic matter, g/kg	--	950.1	954.2	955.3	960.8	973.1	955.0	961.0	970.5	968.5
Crude protein[3], g/kg	--	202.4	171.6	156.1	136.9	133.0	135.5	139.1	114.1	114.1
Total dietary fiber, g/kg		180.3	163.2	161.6	131.1	143.2	133.9	128.1	137.5	119.9
Isoflavones, mg/kg										
Genistein	--	1,375	84.8	1,173	66.0	1,163	48.1	1,010	64.8	753
Daidzein	--	1,064	20.2	923	17.5	928	10.3	809	14.1	605
Glycitein	--	89.4	0.0	75.0	0.0	76.7	0.0	66.7	0.0	49.9
Total isoflavones	--	2,528	105	2,172	83.5	2,172	58.4	1,886	78.9	1,407
Total saponins, mg/kg	--	938	345	852	406	832	250	665	265	571
Total AA, g/kg	--	187.9	172.0	164.5	141.1	132.8	142.8	132.2	123.1	119.1
Indispensable AA, g/kg										
Arginine	--	10.1	9.70	9.10	8.20	7.50	8.30	7.50	7.10	6.70
Histidine	--	5.10	4.80	4.60	3.80	3.60	3.90	3.60	3.40	3.20
Isoleucine	--	8.20	7.50	7.00	6.20	5.70	6.30	5.80	5.40	5.20
Leucine	--	19.3	17.2	16.8	14.0	13.1	13.9	13.3	12.0	11.8
Lysine	--	12.3	10.3	10.0	8.60	8.60	8.90	7.80	7.20	7.10
Methionine	--	3.50	3.20	2.90	2.50	2.30	2.50	2.20	2.00	1.90
Phenylalanine	--	9.40	8.50	8.20	7.10	6.60	7.20	6.60	6.20	5.90
Threonine	--	7.30	6.90	6.60	5.80	5.60	5.60	5.30	5.00	4.80
Tryptophan	--	2.10	2.00	1.80	1.60	1.50	1.50	5.40	1.30	1.40
Valine	--	9.60	9.00	8.50	7.30	6.60	7.30	6.70	6.20	5.90
Dispensable AA, g/kg										
Alanine	--	11.3	10.4	10.1	8.20	7.80	8.20	7.80	7.10	7.00
Aspartic acid	--	16.2	14.9	14.1	12.5	11.6	13.0	11.5	11.1	10.6
Cysteine	--	3.30	3.20	3.00	2.50	2.30	2.60	2.40	2.20	2.10
Glutamic acid	--	32.4	29.6	28.3	25.6	23.6	25.6	23.7	22.3	21.4
Glycine	--	7.90	7.50	7.10	5.90	5.50	6.00	5.60	5.10	4.90
Proline	--	13.6	12.5	12.0	9.30	9.00	9.40	8.90	8.50	8.30
Serine	--	8.20	7.00	6.90	5.70	5.70	5.90	5.40	5.10	4.90
Tyrosine	--	6.20	5.60	5.40	4.40	4.00	4.50	4.30	3.70	3.70

[1]All pigs received allotted treatment diet upon starting -7 DPI. Abbreviations: AA, amino acids; DPI, days post-inoculation; ISF, isoflavones, AA, amino acids.
[2]Missing sample, unable to analyze.
[3]Analyzed CP = total N × 6.25.

Table 4.6. Effects of soy isoflavones and Porcine Reproductive and Respiratory Virus (PRRSV) infection on growth performance of pigs from weaning to market weight[1,2]

	Uninfected	PRRSV-infected			Model
Growth Period	**NEG**	**POS**	**ISF**	**SEM**	***P*-value**
Active infection					
-7 to 0 DPI					
ADG, g/d	112	91.2	98.8	18.0	0.694
ADFI, g/pig/d	435	427	409	38.9	0.862
G:F, g/kg	265	230	248	52.5	0.875
0 to 6 DPI					
ADG, g/d	265[b]	107[a]	128[a]	43.0	0.010
ADFI, g/pig/d	434[a]	537[b]	854[c]	52.8	< 0.001
G:F, g/kg	574[b]	207[a]	142[a]	69.0	0.003
6 to 13 DPI					
ADG, g/d	320[b]	85.3[a]	43.3[a]	62.8	0.022
ADFI, g/pig/d	568[b]	383[a]	503[a]	48.8	0.036
G:F, g/kg	565	298	273	84.8	0.101
13 to 20 DPI					
ADG, g/d	416[b]	258[a]	238[a]	42.9	0.019
ADFI, g/pig/d	856[b]	440[a]	390[a]	88.0	0.005
G:F, g/kg	508	650	797	21.0	0.582
20 to 27 DPI					
ADG, g/d	491	419	421	58.7	0.594
ADFI, g/pig/d	761	791	691	129	0.747
G:F, g/kg	625	567	623	96.9	0.833
27 to 34 DPI					
ADG, g/d	627[b]	404[a]	455[a]	61.4	0.024
ADFI, g/pig/d	1,013	863	943	151	0.683
G:F, g/kg	622	518	500	10.7	0.658
Recovery					
34 to 90 DPI					
ADG, g/d	707	692	648	28.3	0.174
ADFI, g/pig/d	1,569	1,460	1,336	81.2	0.106
G:F, g/kg	450	477	487	16.6	0.278
After viral clearance					
90 to 161 DPI					
ADG, g/d	925	959	967	37.8	0.649
ADFI, g/pig/d	2,591	2,843	2,644	167	0.216
G:F, g/kg	360	342	370	18.8	0.306

[abc]Means without a common superscript letter differ ($P < 0.05$).

[1]Values represent least square means of 17 to 36 pigs across 4-6 replicates per treatment. Pigs were removed from analysis for ADG, ADFI, and G:F if their body weight measurement was deemed as a statistical outlier. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI, day post-inoculation, ADG, average daily gain, ADFI, average daily feed intake, G:F, feed efficiency.

[2]Growth performance was calculated on a pen basis, with each pen representing one experimental replicate containing 1 to 6 pigs per pen. In the event a replicate pen experienced net weight loss over a growth period, they were excluded from the calculation of average feed efficiency of their treatment for that growth period.

Table 4.7. Effects of soy isoflavones and Porcine Reproductive and Respiratory Virus (PRRSV) infection on rectal temperature (°C) of pigs[1]

Day	Uninfected	PRRSV-infected			Model
Post-Inoculation	NEG	POS	ISF	SEM	*P*-value
DPI 0	39.5[b]	39.4[b]	39.0[a]	0.164	0.042
DPI 3	39.3	39.7	39.5	0.164	0.144
DPI 6	39.3[a]	40.0[b]	39.5[a]	0.164	0.005
DPI 10	39.2	39.6	39.3	0.164	0.141
DPI 13	39.3	39.6	39.6	0.164	0.344

[ab]Means without a common superscript letter differ ($P < 0.05$).

[1]Values represent least square means of 12 to 18 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI, day post-inoculation.

Table 4.8. Effects of soy isoflavones and Porcine Reproductive and Respiratory Virus (PRRSV) infection on the erythrogram of weanling pigs[1]

	Uninfected	PRRSV-infected			
Item	**NEG**	**POS**	**ISF**	**SEM**	**Model *P*-value**
RBC, × 10^6 cells/µL					
DPI 0	6.55	6.53	6.68	0.169	0.670
DPI 3	6.22	5.86	5.71	0.169	0.056
DPI 6	6.14[b]	5.71[a]	5.51[a]	0.158	0.004
DPI 13	6.03[c]	5.23[b]	4.73[a]	0.149	< 0.001
Hemoglobin, g/dL					
DPI 0	12.45	12.67	12.69	0.306	0.805
DPI 3	11.70	11.10	10.87	0.306	0.098
DPI 6	11.59[b]	10.64[a]	10.28[a]	0.285	0.001
DPI 13	11.04[b]	9.53[a]	9.07[a]	0.267	< 0.001
Hematocrit, %					
DPI 0	39.83	40.59	40.77	0.965	0.718
DPI 3	36.65	34.90	34.55	0.965	0.193
DPI 6	35.89[b]	33.73[a]	32.93[a]	0.898	0.023
DPI 13	34.78[c]	29.29[b]	26.79[a]	0.845	< 0.001
MCV, fl					
DPI 0	60.58	62.11	61.05	1.01	0.507
DPI 3	58.77	59.64	60.34	1.01	0.479
DPI 6	58.21	59.19	59.83	0.930	0.388
DPI 13	57.50	55.67	56.65	0.870	0.260
MCH, pg					
DPI 0	18.95	19.37	18.99	0.414	0.689
DPI 3	18.78	18.95	18.99	0.414	0.924
DPI 6	18.77	18.63	18.69	0.384	0.961
DPI 13	18.24	18.13	18.21	0.359	0.971
MCHC, g/dL					
DPI 0	31.26	31.19	31.11	0.246	0.882
DPI 3	31.93	31.77	31.46	0.246	0.284
DPI 6	32.23	31.47	31.24	0.229	0.003
DPI 13	31.72[a]	32.14[b]	32.28[b]	0.228	0.159

[abc]Means without a common superscript letter differ ($P < 0.05$).

[1]Values represent least square means of 6 to 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI, day post-inoculation; RBC, red blood cells; MCH, Mean Cell Volume; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration.

Table 4.9. Effects of soy isoflavones and Porcine Reproductive and Respiratory Virus (PRRSV) infection on the leukogram of weanling pigs[1]

	Uninfected	PRRSV-infected			Model
Item	**NEG**	**POS**	**ISF**	**SEM**	***P*-value**
WBC, × 10^3 cells/µL					
DPI 0	13.77	14.73	14.45	1.90	0.930
DPI 3	19.96[b]	13.22[a]	10.71[a]	1.90	0.001
DPI 6	21.29[b]	12.58[a]	15.82[a]	1.76	0.001
DPI 13	23.08[b]	17.43[a]	19.44[a]	1.65	0.034
Neutrophils, % of WBC					
DPI 0	39.54	41.93	44.59	5.17	0.729
DPI 3	51.03	58.97	62.92	5.17	0.196
DPI 6	43.98[a]	50.55[a]	63.67[b]	4.80	0.003
DPI 13	26.55[a]	50.58[b]	61.09[c]	4.80	< 0.001
Band Cells, % of WBC					
DPI 0	0.04	0.46	0.07	1.65	0.976
DPI 3	0.95	1.37	2.93	1.65	0.595
DPI 6	1.01	0.90	2.28	1.53	0.690
DPI 13	0.28	3.17	0.15	1.43	0.144
Lymphocytes, % of WBC					
DPI 0	48.65	47.85	42.39	4.97	0.514
DPI 3	40.49	35.83	26.80	4.97	0.079
DPI 6	47.41[b]	40.18[b]	23.17[a]	4.61	< 0.001
DPI 13	64.41[b]	35.75[a]	29.48[a]	4.61	< 0.001
Monocytes, % of WBC					
DPI 0	11.09	8.66	11.68	1.63	0.265
DPI 3	5.44	2.75	6.71	1.63	0.067
DPI 6	5.44	5.90	6.17	1.52	0.924
DPI 13	5.74	7.08	6.85	1.43	0.728
Eosinophils, % of WBC					
DPI 0	0.67	1.00	1.00	0.586	0.887
DPI 3	1.67	0.82	0.67	0.586	0.383
DPI 6	2.00	1.90	1.73	0.542	0.919
DPI 13	2.38	2.00	1.56	0.507	0.501
Basophils, % of WBC					
DPI 0	0.16	0.14	0.30	0.280	0.867
DPI 3	0.33	0.18	0.00	0.280	0.636
DPI 6	0.27	0.51	0.33	0.260	0.745
DPI 13	0.37	1.00	0.80	0.244	0.132
Neutrophil Count, × 10^3 cells/µL					
DPI 0	5.47	6.24	6.52	1.21	0.786
DPI 3	10.52[b]	7.67[a]	6.49[a]	1.21	0.036
DPI 6	9.11[b]	5.75[a]	9.53[b]	1.12	0.009
DPI 13	6.27[a]	9.52[b]	11.72[b]	1.12	0.002
Band Cell Count, × 10^3 cells/µL					
DPI 0	0.00	0.09	0.01	0.554	0.991
DPI 3	0.22	0.17	0.32	0.554	0.970
DPI 6	0.22	0.10	0.30	0.513	0.941
DPI 13	0.07	1.15	0.06	0.480	0.108

[abc]Means without a common superscript letter differ ($P < 0.05$).

[1]Values represent least square means of 6 to 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI, day post-inoculation; WBC, white blood cells.

Table 4.9. Continued[1]

Item	Uninfected	PRRSV-infected		SEM	Model *P*-value
	NEG	POS	ISF		
Lymphocyte Count, × 10^3 cells/μL					
DPI 0	6.77	6.94	6.00	0.983	0.694
DPI 3	7.91[b]	4.78[a]	3.06[a]	0.983	0.001
DPI 6	10.36[b]	4.32[a]	3.56[a]	0.910	< 0.0001
DPI 13	14.80[b]	6.35[a]	6.20[a]	0.910	< 0.0001
Monocyte Count, × 10^3 cells/μL					
DPI 0	1.53	1.28	1.71	0.334	0.548
DPI 3	1.03	0.44	0.80	0.334	0.230
DPI 6	1.20	0.73	0.99	0.311	0.478
DPI 13	1.34	1.39	1.36	0.292	0.993
Eosinophil Count, × 10^3 cells/μL					
DPI 0	0.08	0.13	0.14	0.106	0.892
DPI 3	0.28	0.12	0.09	0.106	0.351
DPI 6	0.48	0.23	0.27	0.098	0.139
DPI 13	0.57	0.34	0.34	0.091	0.104
Basophil Count, × 10^3 cells/μL					
DPI 0	0.02	0.03	0.05	0.043	0.817
DPI 3	0.05	0.03	0.00	0.043	0.600
DPI 6	0.04	0.05	0.06	0.040	0.934
DPI 13	0.06	0.17	0.10	0.037	0.061

[abc]Means without a common superscript letter differ ($P < 0.05$).

[1]Values represent least square means of 6 to 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI, day post-inoculation; WBC, white blood cells.

Table 4.10. Effects of soy isoflavones and Porcine Reproductive and Respiratory Virus (PRRSV) infection on serum viral RNA (Ct value) in weanling pigs[1]

Day	Uninfected	PRRSV-infected			Model
Post-Inoculation	NEG	POS	ISF	SEM	*P*-value[2]
DPI 0	ND	ND	ND	0.957	1.000
DPI 3	ND	14.50	13.65	0.998	0.358
DPI 6	ND	13.98	15.50	0.998	0.621
DPI 13	ND	17.12	18.71	0.923	0.629

[1]Values represent least square means of 12 to 18 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI, day post-inoculation; ND, non-detectable; Ct, cycle threshold.
[2]*P*-values represented were calculated on $\log^2$ transformed data.

Table 4.11. Effects of soy isoflavones and Porcine Reproductive and Respiratory Virus (PRRSV) infection on peripheral blood T-cell immunophenotypes of weanling pig[1]

	Uninfected	PRRSV-infected			Model
Cellular Outcome, %	**NEG**	**POS**	**ISF**	**SEM**	***P*-value**
Total Lymphocytes					
DPI 13	42.3[b]	20.9[a]	17.2[a]	4.25	< 0.001
DPI 20	50.2	45.1	41.0	4.25	0.234
Mature T-cells[3]					
DPI 13	42.0[a]	49.7[b]	51.5[b]	3.08	0.034
DPI 20	51.1	45.6	51.4	3.08	0.156
Helper T-cells[4]					
DPI 13	24.9	28.5	32.4	2.55	0.069
DPI 20	21.8	20.6	23.5	2.55	0.591
Cytotoxic T-cells[5]					
DPI 13	32.5[b]	23.8[a]	23.3[a]	3.15	0.050
DPI 20	37.0	34.7	33.1	3.15	0.625
Memory T-cells[6]					
DPI 13	11.3[b]	7.05[a]	7.31[a]	1.27	0.023
DPI 20	9.03[a]	11.5[ab]	13.2[b]	1.27	0.046
Helper:Cytotoxic T-cell Ratio[7]					
DPI 13	0.83[a]	1.19[a]	1.53[b]	0.143	0.001
DPI 20	0.64	0.66	0.77	0.143	0.726

[ab]Means without a common superscript letter differ ($P < 0.05$).
[1]Values represent least square means of 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI, day post-inoculation
[3]Percent of total lymphocytes that are positive for cell-surface marker CD3
[4]Percent of CD3-positive lymphocytes that are also positive for cell-surface markers CD4, CD8, or CD4/CD8
[5]Percent of CD3-positive lymphocytes that are also positive for cell-surface markers CD8
[6]Percent of CD3-positive lymphocytes that are also positive for cell-surface markers CD4/CD8
[7]Calculated ratio of percent helper T-cells to percent cytotoxic T-cells

Table 4.12. Effects of soy isoflavones and Porcine Reproductive and Respiratory Virus (PRRSV) infection on serum chemistry parameters of weanling pigs[1]

	Uninfected	PRRSV-infected			Model
Item	**NEG**	**POS**	**ISF**	**SEM**	***P*-value**
Creatinine, mg/dL					
DPI 0	1.06	1.02	0.99	0.068	0.658
DPI 3	0.84	0.92	0.86	0.062	0.517
DPI 6	0.74[a]	0.91[b]	0.90[b]	0.058	0.035
DPI 13	0.65	0.73	0.69	0.055	0.537
BUN, mg/dL					
DPI 0	9.37	9.86	11.6	1.43	0.335
DPI 3	10.9	12.2	11.7	1.31	0.717
DPI 6	10.2	11.4	11.4	1.22	0.686
DPI 13	12.5	14.7	13.7	1.14	0.349
Total Protein, g/dL					
DPI 0	4.71	4.71	4.53	0.143	0.386
DPI 3	4.68[b]	4.25[a]	4.24[a]	0.130	0.017
DPI 6	4.74[b]	4.29[a]	4.18[a]	0.121	0.003
DPI 13	4.79	4.98	4.82	0.113	0.362
Albumin, g/dL					
DPI 0	2.98	3.04	2.95	0.129	0.774
DPI 3	2.93	2.74	2.58	0.118	0.071
DPI 6	2.94[b]	2.52[a]	2.40[a]	0.109	0.002
DPI 13	2.81[b]	2.18[a]	2.19[a]	0.102	< 0.001
Globulin, g/dL					
DPI 0	1.74	1.67	1.58	0.096	0.368
DPI 3	1.76	1.52	1.66	0.088	0.073
DPI 6	1.81	1.77	1.78	0.082	0.927
DPI 13	1.98[a]	2.79[b]	2.63[b]	0.077	< 0.001
Albumin:Globulin Ratio					
DPI 0	1.80	1.84	1.93	0.124	0.693
DPI 3	1.68	1.83	1.56	0.114	0.098
DPI 6	1.66	1.44	1.37	0.101	0.135
DPI 13	1.43[b]	0.82[a]	0.84[a]	0.099	< 0.001
Calcium, mg/dL					
DPI 0	11.8	12.2	11.9	0.693	0.861
DPI 3	14.1[b]	11.2[a]	10.6[a]	0.634	0.0001
DPI 6	14.3[b]	11.1[a]	10.1[a]	0.588	< 0.001
DPI 13	12.7[b]	10.7[a]	11.1[a]	0.551	0.023
Phosphorus, mg/dL					
DPI 0	4.54	4.21	4.19	0.316	0.626
DPI 3	4.20	3.94	3.95	0.290	0.742
DPI 6	4.40	3.80	3.89	0.269	0.194
DPI 13	4.06	4.30	4.38	0.252	0.607
Sodium, mmol/L					
DPI 0	139	140	139	1.29	0.829
DPI 3	137	139	140	1.18	0.183
DPI 6	136	137	136	1.09	0.705
DPI 13	137[b]	133[a]	135[b]	1.02	0.003

[ab]Means without a common superscript letter differ ($P < 0.05$).

[1]Values represent least square means of 6 to 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI, day post-inoculation, BUN, blood urea nitrogen

Table 4.12. Continued[1]

	Uninfected	PRRSV-infected			Model
Item	**NEG**	**POS**	**ISF**	**SEM**	***P*-value**
Potassium, mmol/L					
DPI 0	4.89	5.35	5.33	0.261	0.273
DPI 3	4.90	4.64	4.91	0.241	0.482
DPI 6	5.52[b]	4.95[a]	4.80[a]	0.226	0.040
DPI 13	6.20	6.13	6.09	0.213	0.907
Sodium:Potassium Ratio					
DPI 0	28.5	26.6	26.4	1.26	0.349
DPI 3	28.1	30.4	28.9	1.27	0.212
DPI 6	24.8[a]	27.8[b]	28.2[b]	1.09	0.032
DPI 13	22.4	22.0	22.3	1.03	0.939
Chloride, mmol/L					
DPI 0	97.8	99.2	100.0	0.953	0.183
DPI 3	95.8[a]	99.1[b]	98.6[b]	0.873	0.009
DPI 6	96.4[a]	99.0[b]	98.6[b]	0.810	0.037
DPI 13	100.2[b]	96.9[a]	96.2[a]	0.759	< 0.001
Glucose, mg/dL					
DPI 0	109	114	116	5.30	0.507
DPI 3	108[b]	94.6[a]	90.9[a]	4.84	0.024
DPI 6	99.4[b]	93.4[b]	83.7[a]	4.48	0.048
DPI 13	96.3[b]	74.0[a]	77.6[a]	4.19	< 0.001
Alkaline Phosphatase, U/L					
DPI 0	287	327	251	50.5	0.329
DPI 3	312	386	270	46.4	0.064
DPI 6	392[b]	277[a]	189[a]	43.1	0.004
DPI 13	495[b]	139[a]	92.8[a]	40.4	< 0.001
Aminotransferase, U/L					
DPI 0	39.7	33.2	25.1	9.78	0.410
DPI 3	51.0	61.6	53.7	9.03	0.536
DPI 6	40.8[a]	66.5[b]	68.9[b]	8.45	0.018
DPI 13	65.5	83.6	76.5	7.97	0.191
Gamma-Glutamyl Transferase, U/L					
DPI 0	34.1	34.8	41.2	4.98	0.286
DPI 3	37.9	38.1	41.0	4.63	0.791
DPI 6	44.5	42.0	46.4	4.35	0.631
DPI 13	43.5	49.8	50.0	4.13	0.366
Total Bilirubin, mg/dL					
DPI 0	0.173	0.157	0.183	0.078	0.947
DPI 3	0.123[a]	0.371[b]	0.359[b]	0.071	0.012
DPI 6	0.136	0.225	0.271	0.066	0.329
DPI 13	0.130	0.144	0.108	0.062	0.889
Creatine Phosphokinase, U/L					
DPI 0	652	473	270	264	0.478
DPI 3	1,116	556	428	241	0.077
DPI 6	716	561	425	223	0.654
DPI 13	1,083[b]	841[ab]	381[a]	209	0.034

[ab]Means without a common superscript letter differ ($P < 0.05$).

[1]Values represent least square means of 6 to 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI, day post-inoculation, BUN, blood urea nitrogen

Table 4.12. Continued[1]

	Uninfected	PRRSV-infected			Model
Item	**NEG**	**POS**	**ISF**	**SEM**	***P*-value**
Cholesterol, mg/dL					
DPI 0	62.6	63.9	61.4	5.46	0.906
DPI 3	65.0	59.3	55.4	4.99	0.337
DPI 6	76.4	84.8	73.4	4.62	0.118
DPI 13	75.1[b]	58.3[a]	56.6[a]	4.32	0.003
Glutamate Dehydrogenase, U/L					
DPI 0	3.80[b]	2.89[a]	2.18[a]	0.348	0.001
DPI 3	1.95	1.95	1.80	0.318	0.900
DPI 6	1.63	2.01	2.31	0.294	0.260
DPI 13	2.30	2.25	1.89	0.275	0.440
Total CO_2, mmol/L					
DPI 0	25.0	25.7	24.7	1.33	0.775
DPI 3	28.9	27.5	28.0	1.23	0.722
DPI 6	26.6	26.4	25.3	1.13	0.663
DPI 13	23.9[a]	25.6[ab]	27.4[b]	1.05	0.045
Magnesium, mg/dL					
DPI 0	1.85	1.83	1.83	0.119	0.986
DPI 3	1.83	1.67	1.74	0.109	0.461
DPI 6	1.83	1.67	1.72	0.101	0.400
DPI 13	2.06	1.90	1.98	0.095	0.427
Triglycerides, mg/dL					
DPI 0	41.4	44.6	38.6	7.36	0.713
DPI 3	34.7	29.0	25.5	6.77	0.554
DPI 6	44.5	51.2	49.0	6.31	0.681
DPI 13	65.0	51.0	51.4	5.93	0.121
Anion Gap					
DPI 0	20.7	20.2	20.8	1.54	0.920
DPI 3	17.6	17.1	18.4	1.41	0.689
DPI 6	18.9	16.7	16.6	1.31	0.335
DPI 13	19.5	16.7	17.7	1.23	0.207

[ab]Means without a common superscript letter differ ($P < 0.05$).

[1]Values represent least square means of 6 to 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI, day post-inoculation, BUN, blood urea nitrogen

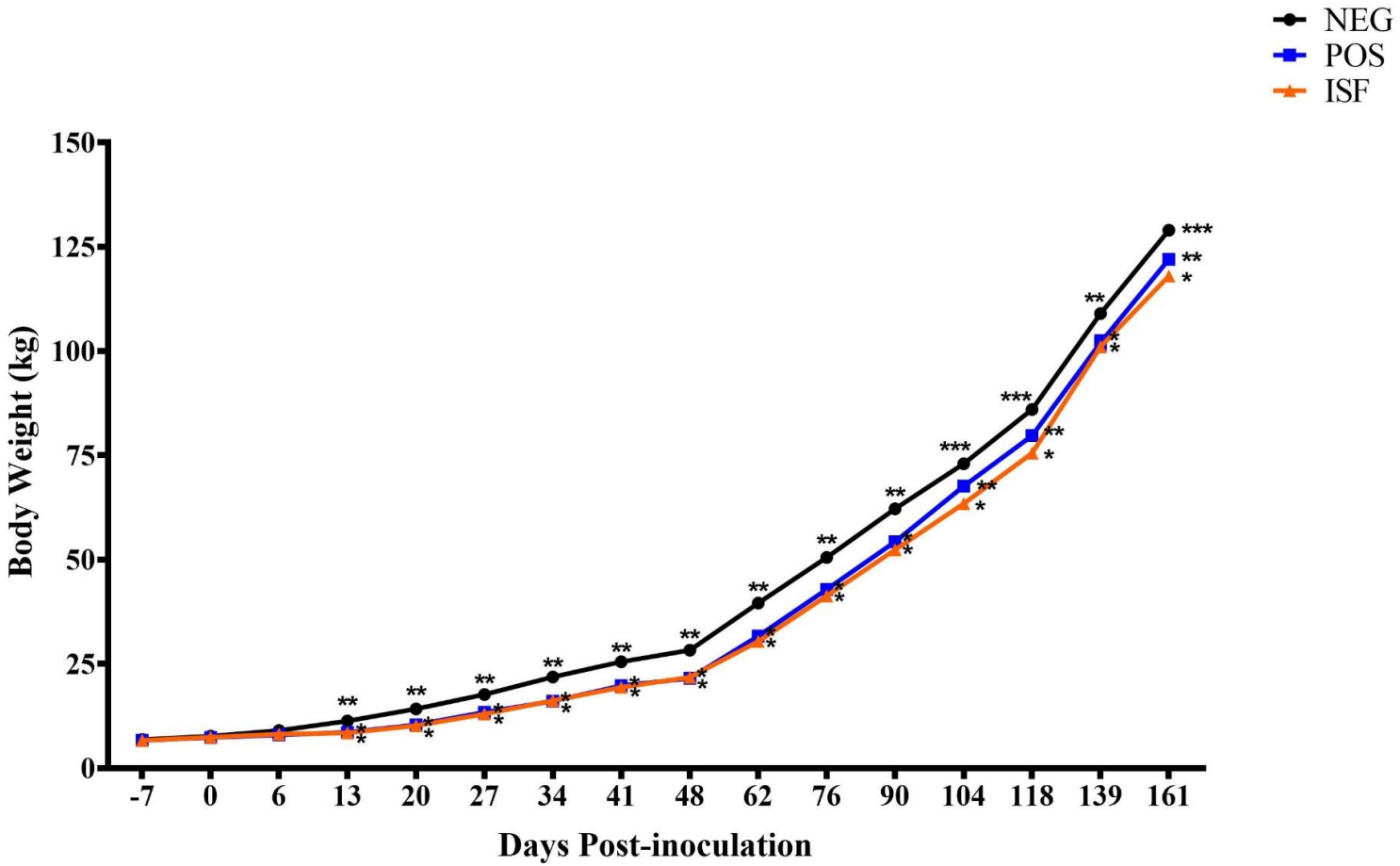


Figure 4.1. Average body weights of pigs by treatment over the course of the entire growth period. Means assigned differing numbers of asterisks (*) are different at $P < 0.05$. Treatment ID: NEG, control diet + uninfected; POS, control diet + PRRSV-infection; ISF, control diet + ISF + PRRSV-infection.

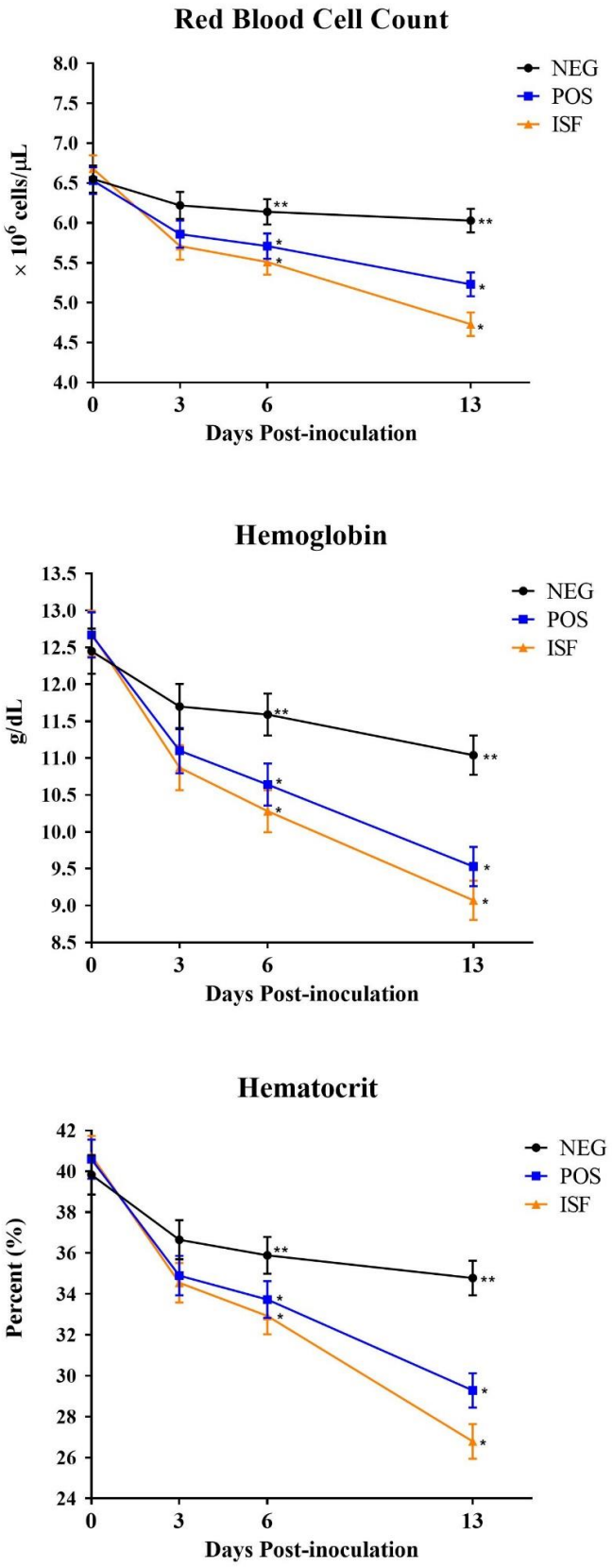
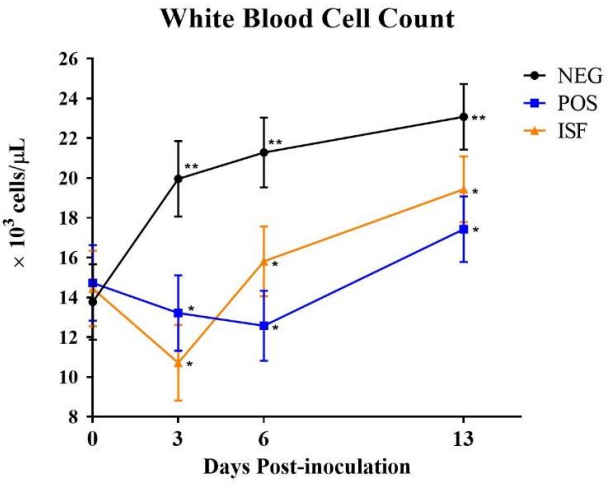
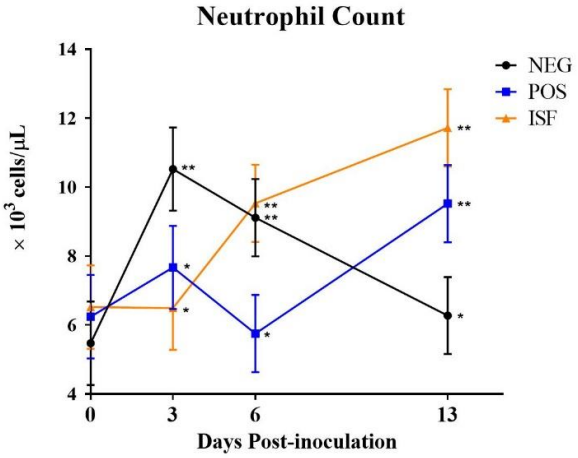


Figure 4.2. Erythrogram results for red blood cell counts of whole blood, hemoglobin concentrations of whole blood, and percent hematocrit of whole blood collected on 0, 3, 6, and 13 days post-inoculation by treatment. Means assigned differing numbers of asterisks (*) are different at $P < 0.05$. Abbreviations: PRRSV, porcine reproductive and respiratory syndrome virus; ISF, isoflavones; Treatment ID: NEG, control diet + uninfected; POS, control diet + PRRSV-infection; ISF, control diet + ISF + PRRSV-infection.





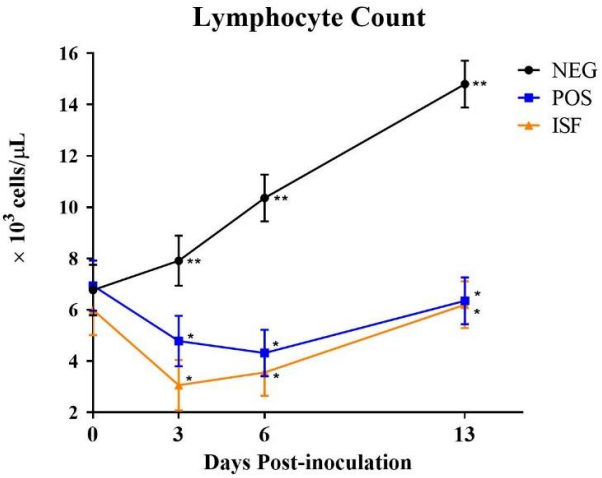
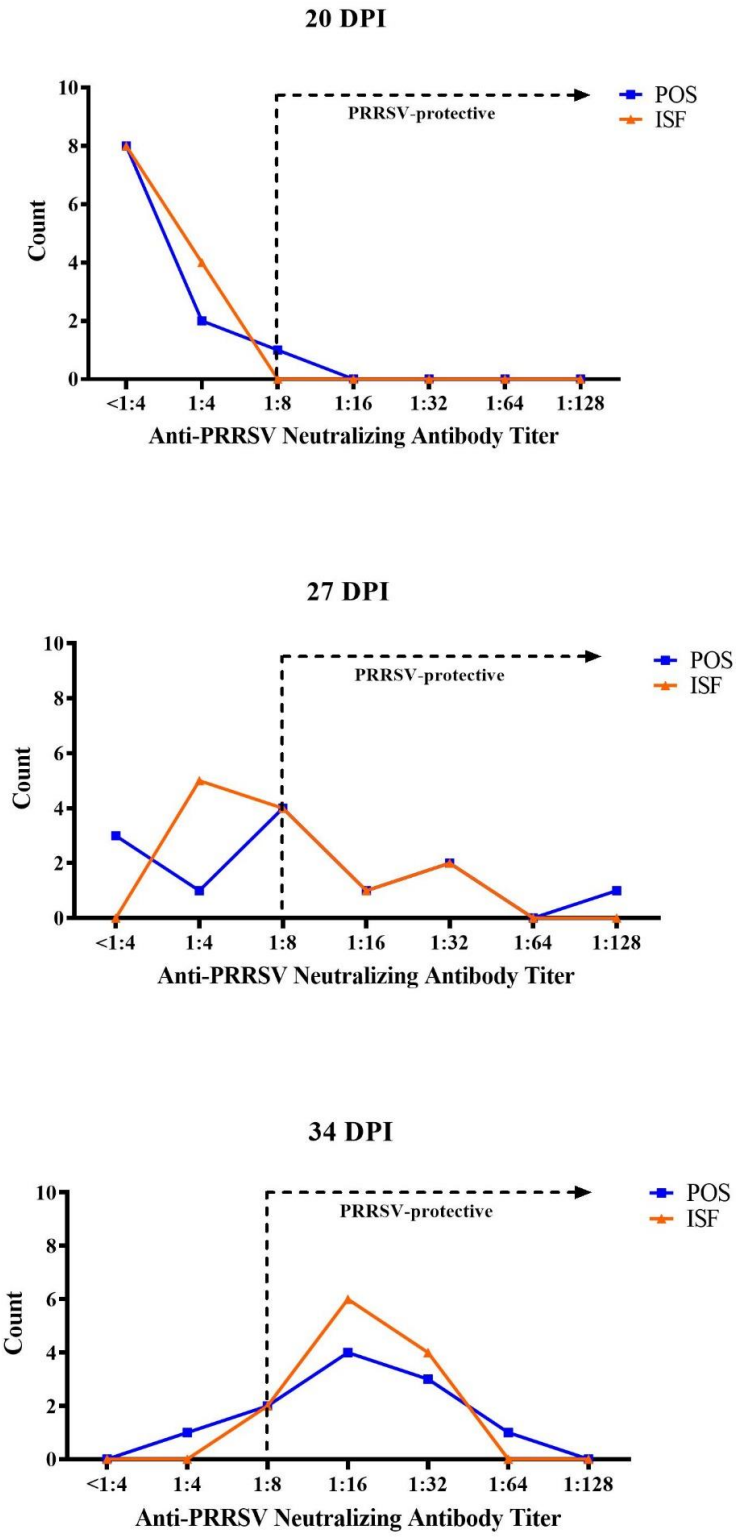


Figure 4.3. Leukogram results for white blood cell counts, raw neutrophil cell counts, and raw lymphocyte cell counts of whole blood collected on 0, 3, 6, and 13 days post-inoculation by treatment. Means assigned differing numbers of asterisks (*) are different at $P < 0.05$. Abbreviations: PRRSV, porcine reproductive and respiratory syndrome virus; ISF, isoflavones; Treatment ID: NEG, control diet + uninfected; POS, control diet + PRRSV-infection; ISF, control diet + ISF + PRRSV-infection.



Figures 4.4. Counts of individual pigs within treatment with detectable anti-PRRSV neutralizing antibody titers and associated titer result at 20, 27, and 34 DPI. Antibody titers were measured in 12 pigs per treatment and the same individual pigs were used for sample collection when possible. NEG treatment was omitted from these figures as they never tested positive for anti-PRRSV antibodies across all the time points measured. Abbreviations: PRRSV, porcine reproductive and respiratory syndrome virus; DPI, days post-inoculation.

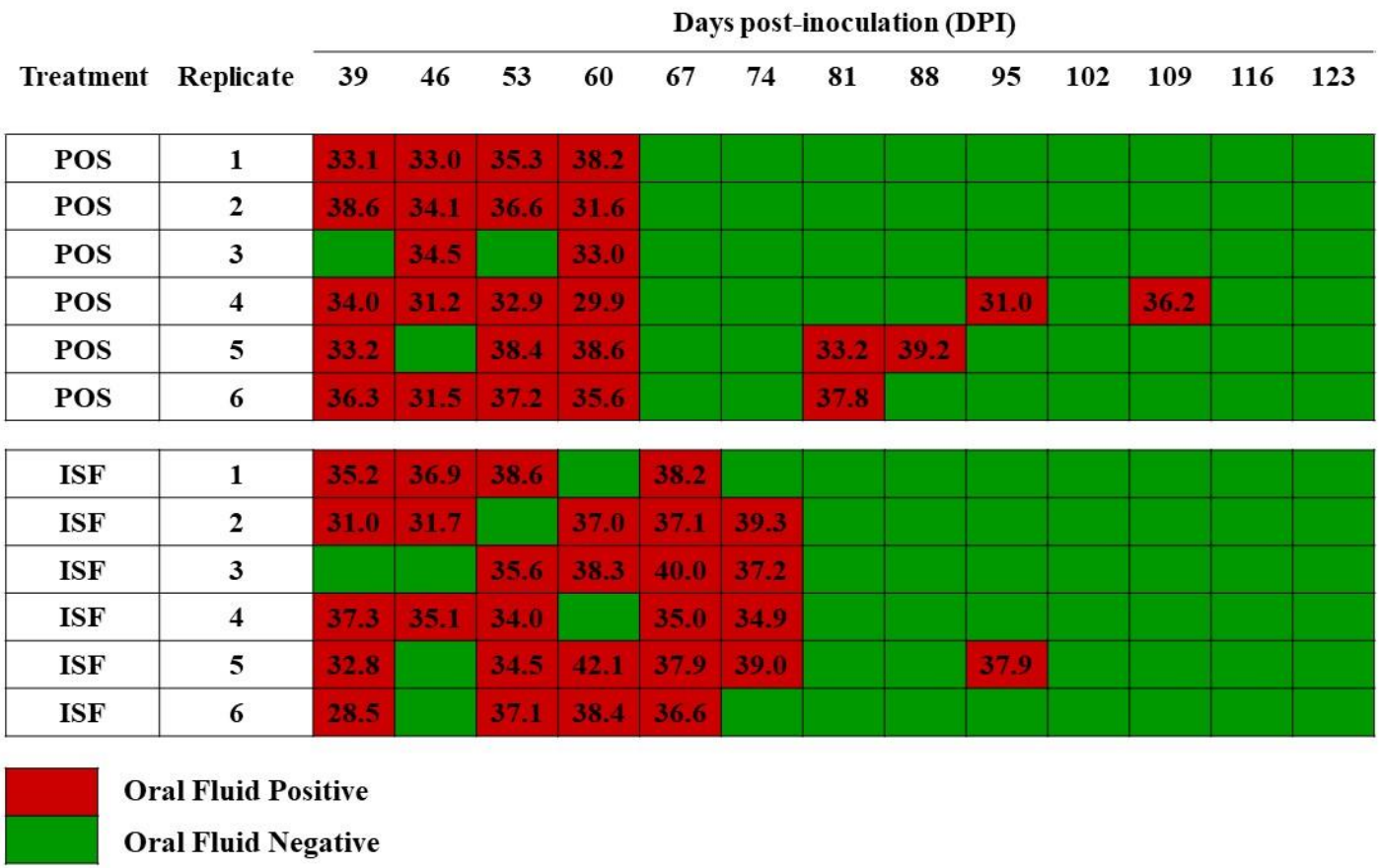


Figure 4.5. Visual representation of PRRSV status on pen basis by PRRS viral antigen via qRT-PCR in group-based oral fluids from 39 DPI onward. Oral fluids were collected from up to 6 pens per treatment and collection was discontinued for an individual pen following 2 consecutive weeks of negative test results. The NEG treatment was omitted from this figure as no NEG pens tested positive for PRRS viral RNA over the course of the experiment. Values represent Ct value of submitted samples. A Ct value ≤ 38.0 was considered a positive result and a Ct value of 38.0-45.0 is considered suspect. Abbreviations: PRRSV, porcine reproductive and respiratory syndrome virus; ISF, isoflavones. Treatment ID: POS, control diet + PRRSV-infection; ISF, control diet + ISF + PRRSV-infection.

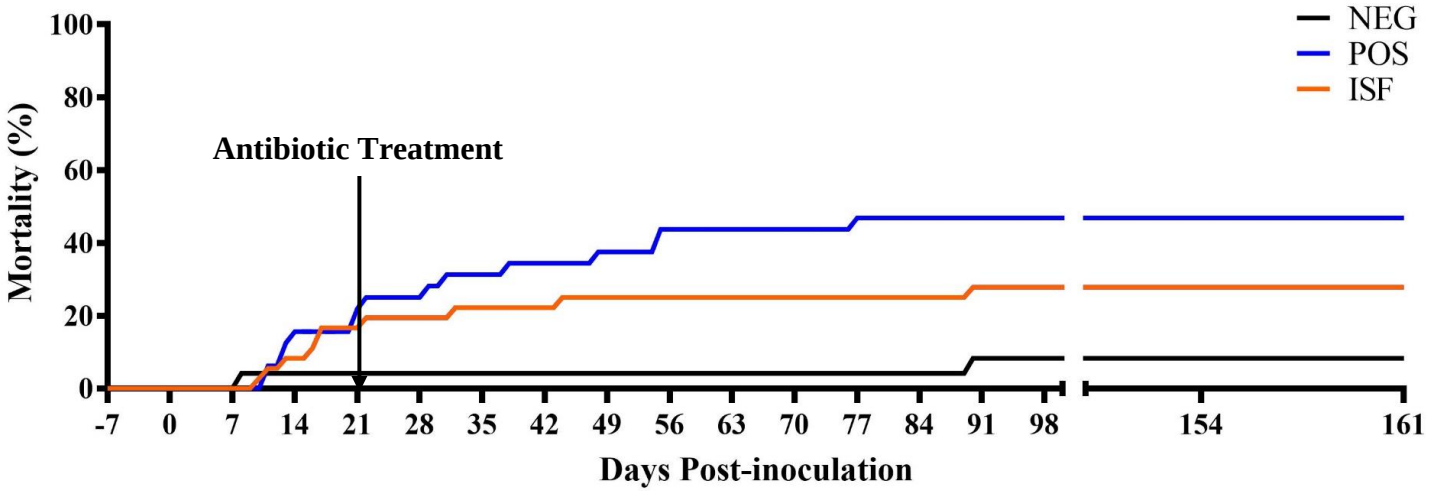


Figure 4.6. Percent mortality within treatment across the entire experiment depicted in reference to the inoculation schedule. Percent mortality is out of 24 pigs total for the NEG treatment and 36 pigs total for both POS and ISF treatments. Abbreviations: PRRSV, porcine reproductive and respiratory syndrome virus; ISF, isoflavones. Treatment ID: NEG, control diet + uninfected; POS, control diet + PRRSV-infection; ISF, control diet + ISF + PRRSV-infection.

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CHAPTER 5: ALTERATIONS OF FECAL MICROBIOME CHARACTERISTICS BY DIETARY SOY ISOFLAVONE INGESTION IN GROWING PIGS INFECTED WITH PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is an economically important disease and ingestion of soy isoflavones (ISF) may benefit PRRSV-infected pigs due to demonstrated anti-inflammatory and anti-viral properties. The objective of this study was to quantify long-term effects of ISF consumption on fecal microbiome characteristics under disease challenge. In total, 96 weaned barrows were group-housed in a Biosafety Level-2 containment facility and allotted to 1 of 3 experimental treatments that were maintained throughout the study: non-infected pigs receiving an ISF-devoid control diet (NEG, *n*=24), and infected pigs receiving either the control diet (POS, *n*=36) or that supplemented with total ISF in excess of 1,600 mg/kg (ISF, *n*=36). Following a 7-day adaptation, pigs were inoculated intranasally with either a sham-control (PBS) or live PRRSV (1×10^5 $TCID_{50}$/mL, strain NADC20). Fecal samples were collected from 48 individual pigs at pre-infection (-2 days post-inoculation, DPI), peak-infection (10 DPI), post-infection (144 DPI) time-points. Extracted DNA was used to quantify fecal microbiota profiles via 16S bacterial rRNA sequencing. Differences in bacterial communities among diet groups were evaluated with principal co-ordinate analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA) using UniFrac distance matrices. Both unweighted and weighted UniFrac distances using QIIME 2. All other data were analyzed by one-way ANOVA performed on square root transformations using R. Across all time-points, only minimal differences were observed due to ISF alone. The most notable difference observed was decreased relative abundance of Actinobacteria at 144 DPI between non-infected and

infected treatments ($P < 0.05$), which is consistent with various dysbioses observed in other disease models. Our findings indicate that differences present were mainly due to PRRSV-infection alone and not strongly influenced by diet, which implies previously observed performance benefits conferred by dietary ISF are not likely due to changes in microbiome composition.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) continues to be one of the leading pathogens with high economic impact in modern swine production (Holtkamp et al., 2013). Due to its prevalence and a lack of highly effective vaccines (Loving et al., 2015), nutritional intervention or management strategies are of high interest for PRRSV mitigation due to relatively low cost and ease of implementation. Previously it has been demonstrated that increased levels of soybean meal in diets fed to pigs under immune stress reduces adverse effects of illness and may improve growth performance (Boyd et al., 2010; Rochell et al., 2015). However, it remains unclear if improved growth performance in those studies were due to differences in dietary bioavailable amino acid content or the presence of other dietary components. Among these components, soy isoflavones (**ISF**) are naturally occurring flavonoid compounds that possess anti-inflammatory and anti-oxidative properties (Smith and Dilger, 2018). Under PRRSV-challenge, weaned pigs that received dietary ISF have previously exhibited improved immune responses to PRRSV by improved growth performance, reduce viral load, and improved adaptive immune cell responses by measurements of effector T-cell proportions during peak infection periods (Greiner et al., 2001; Smith et al., 2019). More recently, when ISF were fed to growing pigs infected with PRRSV over the entire growth period

from weaning to market, dietary ISF improved PRRSV clearance during recovery and reduced pathogen-related mortality by approximately 50% (Smith et al., 2020).

In light of these effects on the response to PRRSV-infection in an *in vivo* model, it is unclear if dietary ISF and their metabolites are producing these effects directly following absorption into the body or if these effects are due to alterations of the gastrointestinal microbiome. While it is fairly well accepted that the gastrointestinal microbiome can influence systemic immune system function through the "gut-lung axis" (Collado et al., 2018), there is also evidence that soy ISF can influence the composition of the gastrointestinal microbiome (Nakatsu et al., 2014; Huang et al., 2016; Paul et al., 2017; Vázquez et al., 2017; Kolátorová et al., 2018). These alterations may impact modulation of immune function and thus systemic response to infections. For those reasons, the objective of this experiment was to evaluate how consumption of soy ISF by PRRSV-infected pigs affected the longitudinal composition of the gastrointestinal microbiota throughout the entire growth period. Our hypothesis was that both PRRSV-infection status and consumption of ISF would result in detectable differences in global microbiome composition in growing pigs.

Materials and Methods

The protocol for this experiment was approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of the University of Illinois at Urbana-Champaign.

Animal Husbandry and Experimental Design

This experiment is a continuation of the one described in Chapter 4 of this dissertation and utilized the same animals for sample collection. In total, ninety-six weanling pigs (96 barrows; 6.77 ± 0.94 kg initial body weight) were obtained from a PPRSV-negative, non-PRRSV

vaccinated commercial herd (1050 Cambro genetics; Carthage Veterinary Service, Ltd.) and group-housed at the Innovative Swine Solutions Veterinary Research Facility (**ISS-VRF**), a Biosafety Level 2 production containment facility in Champaign, IL. Pigs were managed at this facility through market weight (approximately 27 weeks of age, 118-129 kg average body weight) and then transported to the Meat Science Laboratory at the University of Illinois for slaughter. The ISS-VRF consisted of 4 individual production rooms (9.1 m x 4.3 m) with 4 pens (up to 6 pigs per pen) available to house pigs in each room. Each room was equipped with a high-efficiency particulate air filtration system, plastic slotted flooring appropriate for market-sized pigs, and each pen provided appropriate floor space for pigs taken to market weight. Lighting and ambient temperature was maintained as appropriate for life stage.

Upon entry to the facility, pigs were weighed and uniformly allotted to 1 of 3 experimental treatments [n = 24 for non-PRRSV-infected controls (**NEG**), n = 36 each for PRRSV-infected controls (**POS**) and PRRSV-infected receiving soy isoflavones (**ISF**)] by completely randomized design based on initial body weight. All replicates of the NEG treatment (6 pigs per replicate, 4 replicates total) were housed in a single room to account for biosecurity while replicates for POS and ISF treatments (6 pigs per replicate, 6 replicates total) were equally represented in the remaining three rooms at ISS-VRF. Two levels of supplemental ISF (none vs. ≥1,600 mg/kg Novasoy400; ADM, Decatur, IL) constituted the total of two dietary treatments (**Table 5.1**). The ISF concentrations used in the ISF treatment were reflective of those typical for a commercially relevant corn-soybean meal diet fed to pigs with approximately 20% soybean meal inclusion (USDA-ARS, 2016). Both diets contained soy protein concentrate (**SPC**) as a protein source, as this ingredient is practically devoid of soy ISF. Experimental diets were formulated to be isocaloric and, with the exceptions of corn and the isoflavone-enriched product,

were identical in ingredient and nutrient composition. Experimental diets were fed over 7 feeding phases including nursery phases 1 and 2 (**N1-N2**) and finisher phases 1-5 (**F1-F5**). For additional information regarding experimental diet formulations and nutrient composition, please refer to Chapter 4 of this dissertation.

Following a 1-week adaptation period to experimental diets [days post-inoculation (**DPI**) -7 to 0], blood was collected from 2 pigs per pen to establish 0 DPI baseline measurements and to ensure that a representative number of pigs were confirmed PRRSV-negative at experiment initiation. Immediately following blood collection, pigs were administered via intranasal inoculation either 2 mL of a 2% fetal bovine serum + phosphate-buffered saline solution (sham-control) or 1.0×10^5 50% tissue culture infective dose of PRRS virus (strain NADC20, courtesy of Dr. Federico Zuckermann, University of Illinois, Urbana, IL).

Fecal Sample Collection

Individual pigs served as the experimental unit for all fecal microbiota outcomes described herein. Fecal samples were collected manually by trained personnel with a clean fecal loop from up to 3 pigs per pen (12-18 pigs/treatment, same pigs collected at each time-point when possible) on -2, 10, and 144 DPI to represent baseline, peak infection, and post-PRRSV clearance time-points. Samples were stored at -80°C pending DNA extraction.

Fecal Bacterial DNA Extraction and Analyses

For DNA extraction, 180-220 mg of feces from individual samples was homogenized using MP Biomedicals Lysing Matrix E 2 mL tubes (MP Biomedicals, Santa Ana, CA). DNA was extracted from homogenized samples using the QIAamp FastDNA Stool Mini Kit (Qiagen, Valencia, CA) per manufacturer instructions and concentrations of isolated DNA were quantified using a Nanodrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Quality

of the DNA was checked on a 1% agarose gel following staining with ethidium bromide. When at sufficiently high quality, individual samples were then diluted to 20 ng/µL using RNAse/DNAse-free water and 15 µL of diluted DNA was transferred to a 96-well plate for submission to the University of Illinois High-Throughput Sequencing and Genotyping Unit at the Roy J. Carver Biotechnology Center for polymerase chain reaction amplification and sequencing of 16S bacterial rRNA genes for microbial profiling.

Statistical Analyses

Differences in bacterial communities among diet groups were evaluated with principal co-ordinate analysis (**PCoA**) and permutational multivariate analysis of variance (**PERMANOVA**) using UniFrac distance matrices. Based on both unweighted and weighted UniFrac distances using QIIME 2 (2019.4) (Caporaso et al., 2011). All other data were analyzed by one-way ANOVA performed on square root transformations using R (version 3.5.4, Vienna, Austria) and sliced by DPI. Please note that due to greater loss in mortality than expected (Smith et al., 2020), several animals were not able to be sampled at each time-point. Thus, we chose to analyze the microbial data individually at each time-point, rather than across time-points. Post-hoc mean separation with a Tukey adjustment was performed if the ANOVA resulted in a *P*-value < 0.05. Mean separation was performed by main effect of treatment and significance was accepted at $P \leq 0.05$. Only bacterial phyla and genera with at least one mean with relative abundance >0.3% and > 1.0%, respectively, were interpreted and summarized in data tables. Data are presented as least-squares means with pooled standard errors of the mean (**SEM**). For relative abundance microbiota outcomes, data are presented as raw least-square means plus SEM, along with *P*-values reflective of the square root transformed values.

Results

For the sake of clarity, measures of alpha diversity (i.e., species richness or number of distinguishable taxa within an individual sample) and beta diversity (i.e., difference between global microbial communities or differences in taxonomic abundance profiles between individual samples) are discussed separately, with beta diversity being discussed first. Please note that some of the bacterial genera detected through our analyses are newly identified and have not yet been fully classified, so they may be identified by higher taxonomic level names, particularly at the family level. In those cases, the bacterial genera names are preceded by the letter "F" in parentheses, indicating that it is associated with a specific taxonomic family.

Beta and Alpha Diversity Measures

Beta diversity was evaluated by PCoA and PERMANOVA using both unweighted and weighted UniFrac distances matrices and are represented by experimental time-point in **Figure 5.1**. During the pre-infection period (-2 DPI), PERMANOVA analysis revealed that overall bacterial composition in fecal samples did not differ between treatments ($P > 0.05$). However, at peak infection (10 DPI), PERMANOVA analysis revealed that overall bacterial composition in fecal samples differed (unweighted, $P = 0.012$; weighted, $P = 0.02$) between non-infected (NEG) and infected treatments (POS and ISF); the two infected treatments did not differ from each other. By the post-infection period, PERMANOVA analysis revealed that overall bacterial composition in fecal samples differed, but only for unweighted UniFrac measures (unweighted, $P = 0.003$; weighted, $P = 0.172$).

Alpha diversity was evaluated using operational taxonomic units (OTU) at the phyla and genera level. Differences in relative abundance of bacteria at these taxonomic levels is discussed in the following section. Visualization of variation in phyla relative abundance among individual

samples across sampling time-points can be found in **Figure 5.2**. Additionally, heat maps depicting the differences in relative abundance of both common (> 1.0%) and rare (< 1.0%) genera among treatments across sampling time-points can be found in **Figures 5.3** and **5.4**, respectively.

Relative Abundance of Bacteria at the Phyla and Genera Level

At the pre-infection period (-2 DPI), 8 phyla and 30 genera were detected at or above our interpretation thresholds (0.3% and 1.0%, respectively). There were no differences in relative abundance at the phyla level between treatments at the pre-infection time-point (**Table 5.2**). At the genera level, the NEG treatment had a greater ($P < 0.05$) relative abundance of *Roseburia* bacteria than POS and ISF treatments (**Table 5.3**).

At peak infection (10 DPI), 6 phyla and 26 genera were detected at or above our interpretation thresholds. At the phyla level, the ISF treatment had greater ($P < 0.05$) relative abundance of Proteobacteria bacteria compared with the NEG treatment, but did not differ from the POS treatment (**Table 5.4**). At the genera level, there were 5 differences detected among experimental treatments (**Table 5.5**). The NEG treatment had greater ($P < 0.05$) relative abundance of *Prevotella 9* compared with POS and ISF treatments and greater ($P < 0.05$) relative abundance of *(F) Prevotellaceae Unassigned* and *Anaerovibrio* compared with the ISF treatment only, with the POS treatment exhibiting intermediated relative abundances for those genera. The ISF treatment had greater ($P < 0.05$) relative abundance of *Prevotellaceae NK3B31 group* compared with the NEG treatment, with the POS treatment exhibiting an intermediate relative abundance. Alternatively, the ISF treatment had lower ($P < 0.05$) relative abundance of *Dialister* compared with the NEG treatment, again with the POS treatment exhibiting an intermediate relative.

At the post-infection time-point (144 DPI), 7 phyla and 27 genera were detected at or above our interpretation thresholds. At the phyla level, there was a difference observed for Actinobacteria, with the NEG treatment expressing greater ($P < 0.05$) relative abundance compared with POS and ISF treatments, though the total relative abundance of this phylum across treatments was low (**Table 5.6**). At the genera level, there were 7 differences detected among experimental treatments (**Table 5.7**). The NEG treatment had greater ($P < 0.05$) relative abundance of *CAG-873* and *Prevotellaceae UCG-004* compared with both the POS and ISF treatments. Both the NEG and POS treatment had greater ($P < 0.05$) relative abundance of *Clostridium sensu stricto 1* compared with the ISF treatment. The NEG treatment had greater ($P < 0.05$) relative abundance of *Ruminococcaceae UCG-002* and *Lachnospiraceae XPB1014 group* compared with the POS treatment, with the ISF treatment exhibiting an intermediate relative. The ISF treatment had greater ($P < 0.05$) relative abundance of *(F) Lachnospiraceae Unassigned* and *Ruminococcaceae UCG-014* compared with the NEG treatment, with the POS treatment exhibiting an intermediate relative abundances.

Discussion

In the live-animal phase of this study (Smith et al., 2020), ISF consumption over the entire growth period resulted in earlier complete viral clearance and an approximately 50% reduction in pathogen-related mortalities in pigs infected with PRRSV, though there were no growth performance benefits observed. Those results suggest that when included at levels that are typical for standard commercial swine diets, soy ISF may support the immune response to PRRSV, particularly in the presence of severe secondary bacterial infections. While it is understood that ISF can interact directly with cell signaling cascades utilized by the immune system (Smith and Dilger, 2018), within the context of this live-animal model it was unclear

whether such beneficial health effects were due to direct action of ISF in circulation or if changes to the gastrointestinal microbiome may be contributing to these systemic effects.

Regarding the role of the gastrointestinal microbiome in immune function, the "gut-lung axis" is a well-documented phenomena that represents the bi-directional communication between microbial populations of the gastrointestinal and respiratory tracts (Collado et al., 2018). In general, the gastrointestinal microbiome has been shown to play a role in the development of innate immunity (e.g., maintenance of homeostasis, epithelial function at mucosal surfaces), T-cell differentiation, modulation of local and systemic inflammation, and regulate homeostasis of the adaptive immune response (Kau et al., 2011; Hooper et al., 2012; Hauptmann and Schaible, 2016; Niederwerder, 2017). These effects are not only driven by the composition of microbial populations in the gastrointestinal tract (**GIT**), but also through the production of different metabolites through fermentation dependent on predominant species present. These metabolites, most notably short-chain fatty acids, have been shown to not only have local effects on mucosal immune and barrier function in the intestinal tract, but also influence the development of peripheral immune cells such as dendritic cells and B-cells, thus impacting their overall function (Collado et al., 2018). When considering immune function at the level of the respiratory tract, it has been suggested that the GIT and respiratory tract share a common mucosal response, which involves effects of the gastrointestinal microbiome on mucosal immunity driving immune responses at distal mucosal sites (Mcghee and Fujihashi, 2012; Hauptmann and Schaible, 2016; Date et al., 2017). In practice, several dysbioses (i.e., microbial imbalances or maladaptation on or inside the body) in the gastrointestinal microbiota have been associated with lung disorders and respiratory infections in humans, including viral pathogens such as influenza virus (Collado et al., 2018). Within respiratory viral pathogen disease models, microbiome composition and

diversity has been shown to be directly associated with clinical outcomes, including disease progression, airway inflammation, immune response, and morbidity (Niederwerder, 2017; Ober et al., 2017).

This relationship between respiratory infections and gastrointestinal dysbioses are also observed in swine respiratory disease models (Niederwerder et al., 2016; Niederwerder, 2017; Ober et al., 2017; Niederwerder et al., 2018). Considering disease models utilizing PRRSV, microbial composition has been linked to differences in pig performance following pathogenic challenge. In a PRRSV and porcine circovirus type 2 (**PCV2**) co-infection model in weanling pigs, researchers found that pigs with the best clinical outcomes had greater fecal microbial diversity than pigs with the worst clinical outcomes. In the worst clinical outcomes group, pigs experienced delayed peak PRRSV-infection with prolonged and increased viremia, higher peak PCV2 viremia levels (non-significant), and increased serum concentrations of *Bacillus cereus*, suggesting these animals experienced a more severe systemic infection and compromised mucosal integrity. Regarding microbiome differences observed, in addition to differences in overall measures of microbiome diversity, pigs in the worst clinical outcomes group notably had no *Escherichia coli* detected in their fecal samples, which has been found to influence feed efficiency and growth performance in growing pigs (Niederwerder et al., 2016). Continuation of the same research found that even early microbiome properties (i.e., composition and diversity prior to disease challenge) influenced performance outcomes of PRRSV/PCV2 co-infected pigs, suggesting that the effects of the gastrointestinal microbiome on immune function extends beyond current GIT conditions. Following subclinical infection, there was a clear divergence between high and low growth rate groups during the mid-to-late infection period, with pigs in the high growth rate group exhibiting reduced viral replication rates and decreased lung lesion

severity. Like previous findings from this group, pigs in the high growth rate group had increased fecal microbial diversity (Ober et al., 2017). In general, a common finding across these studies and other respiratory disease models is increased global microbial diversity at the level of the gastrointestinal tract being associated with improved clinical outcomes in infected individuals.

Within our own experiment, we observed differences in beta diversity or global microbial diversity at peak-infection and post-infection time-points (10 and 144 DPI) between experimental treatments. These differences were most notable at our peak-infection time-point, with significance achieved for both weighted and unweighted UniFrac distance matrices, and appear to mainly be due to PRRSV-infection as infected treatments did not differ from one another. At the post-infection time point, there was an effect of treatment on beta diversity with the NEG treatment differing from our infected treatments, but only for unweighted UniFrac distance matrices. These differences are in line with the PRRSV co-infection models described above, but suggest that at the inclusion level utilized for this experiment ISF do not result in dramatic shifts of global microbial diversity in the GIT.

While we observed differences that appeared to be due to PRRSV alone, alpha diversity or relative abundance at both the phyla and genera levels were not strongly influenced ISF supplementation. Regardless, a vast majority of differences in relative abundance occurred so at post-inoculation time-points. At the peak-infection time-point, we detected a single difference at the phyla level between the NEG and ISF treatment for Proteobacteria with the ISF treatment showing greater relative abundance. Interestingly, increased abundance of Proteobacteria has been associated with improved clinical outcomes under swine pathogen challenge (Niederwerder et al., 2016; Smith et al., 2020). However because the ISF treatment did not differ from the POS

treatment it is unclear whether this increased relative abundance contributed to any of the performance differences observed in the live animal phase of this experiment. At the genera level, reduced relative abundance of *Prevotella 9, (F) Prevotellaceae Unassigned,* and *Anaerovibrio* genera appear to be mainly driven by PRRSV-infection. Among these findings, the difference in *Prevotella 9* abundance is most notable due to its reduction from nearly 20% in non-infected control pigs to less than 10% in infected pigs. However, knowing the specific function of this *Prevotella* genera is difficult due to genetic and overall microbiota differences in the host. *Prevotella,* while having been identified as potentially beneficial microbes, are also linked to inflammatory conditions such as colitis and arthritis under immune suppressive states (Dillon et al. 2016). Based on those findings, we would have expected a relationship for *Prevotella* abundance with a PRRSV-infection model opposite to what was observed. Additionally, previous nutrition studies in humans suggest that effects of single diet ingredients on *Prevotella* are not easily predicted (Kovatcheva-Datchary et al. 2015). Regarding dietary supplementation, ISF increased the relative abundance of the *Prevotellaceae NK3B31 group* and decreased the relative abundance of *Dialister* compared with the NEG treatment. Of these two genera quantified in intestinal dysbioses models, *Prevotellaceae* species has been found to be a driver of chronic inflammation at the level of the GIT. In the context of our model, one could argue that ISF in the presence of a PRRSV-infection support the growth of this particular bacteria species (Palm et al., 2015).

Moving to the post-infection time-point, we detected a single difference at the phyla level for relative abundance of Actinobacteria between our NEG treatment and our infected treatments, with PRRSV-infection appearing to decrease the relative abundance of this bacteria. Reduction in the relative abundance of Actinobacteria has been linked to various dysbioses for

which changes typically represent a loss of microbial richness that may directly impact amino acid synthesis, cell junction integrity, and inflammatory responses (Rinninella et al., 2019). At the genera level, reduced relative abundance of the *CAG-873* and *Prevotellaceae UCG-004* genera appeared to be driven by PRRSV-infection alone. The reduction in *Prevotellaceae UCG-004* specifically here is unexpected as other *Prevotellaceae* genera measured in this experiment demonstrated the opposite relationship. Regarding ISF supplementation, ISF appeared to increase the relative abundance of *(F) Lachnospiraceae Unassigned* and *Ruminococcaceae UCG-014* genera relative to the NEG treatment and decreased the relative abundance of *Clostridium sensu stricto 1* genera relative to both the NEG and POS treatments. Alternatively, the ISF treatment exhibited intermediate relative abundance of *Ruminococcaceae UCG-002* and *Lachnospiraceae XPB1014 group* genera compared with the NEG and POS treatments. In previous PRRSV-challenge studies, pigs demonstrating improved performance exhibited increased species diversity in the *Ruminococcaceae* family, which was previously associated with improved growth performance (Ober et al., 2017). In the context of our model, it may be that ISF in the presence of a PRRSV-infection supports the growth of certain *Ruminococcaceae* bacteria within the GIT, though it is difficult to speculate what the specific role these individual genera play in the global response to PRRSV-infection. Increased relative abundance of *Lachnospiraceae* bacteria have been associated with gastrointestinal dysbioses, specifically irritable bowel syndrome in humans (Rinninella et al., 2019), though within our model there appear to be inconsistent effects of ISF supplementation on its presence under PRRSV-challenge.

Though a number of differences in relative abundance were detected within our model based on our interpretation criteria, interpretation of these findings must be done conservatively.

It is important to note that the majority of differences detected at the genera level and involved bacterial genera that on average had a relative abundance of less than 3% (range: 0.16% to 18.5% across all treatments). Additionally, many of these genera are either newly identified or not well described regarding their role in swine specifically. Due to that limitation and the relatively low abundance of these bacteria, it is difficult to make conclusions on their specific role in the clinical response to PRRSV-infection within our model.

While our model did result in changes to global microbial diversity, the magnitude of those differences appear to be less than previously observed in PRRSV-challenge models (Niederwerder et al., 2016; Ober et al., 2017). This is particularly noticeable when referencing visualizations of phyla and genera relative abundance across our experimental time-points (**Figures 5.2-5.4**); differences between experimental treatments are not particularly robust, especially when considering pre- and post-infection time-points. That could be due to differences in how global diversity was measured between our experiment and previous models or simply unaccounted for differences in viral strain properties and influence on the GIT. Additionally, while we can appreciate that there are noticeable visual differences in relative abundance distributions across experimental time-points (e.g., phyla relative abundance at -2 DPI vs. 144 DPI), we analyzed each experimental time-point individually, which prevented us from taking the effect of time into account on individual microbiome composition.

Implications

In conclusion, there were few differences detected at either the phyla or genera level between treatments regardless of time point. Of the differences observed, the reduction in the relative abundance of Actinobacteria phylum during the post-infection period (144 DPI) may be of interest due to its association with dysbioses within other disease models. Our observations

suggest that most of the differences present were likely due to PRRSV infection alone and not strongly influenced by diet, which implies that the performance benefits conferred by dietary isoflavones in our live animal phase, such as reduced mortality, were not likely due to changes in microbiome composition or function.

Implications

In conclusion, there were few differences detected at either the phyla or genera level between treatments regardless of time point. Of the differences observed, some have been previously associated with gastrointestinal dysbioses and differences in pig performance under respiratory disease challenge. Our observations suggest that most of the differences present were likely due be driven by PRRSV-infection and not strongly influenced or inconsistently influenced by diet, which implies that the performance benefits observed within infected treatments conferred by dietary isoflavones in the companion study, such as reduced mortality, were not likely due to changes in microbiome composition or function.

Tables and Figures

Table 5.1. Experimental treatments[1]

Treatment	Dietary Treatment	Infection Status
NEG	Control diet	Uninfected
POS	Control diet	PRRSV-Infected
ISF	Control diet + ISF[2]	PRRSV-Infected

[1]Abbreviations: PRRSV, porcine reproductive and respiratory syndrome virus; ISF, isoflavones.
[2]ISF provided by Novasoy 400 (ADM, Decatur, IL) at >1,600 mg/kg complete diet across all feeding phases.

Table 5.2. Percent relative abundance of bacterial phyla detected in fecal samples at -2 DPI[1,2]

	Treatment			
Bacterial phyla[3]	**NEG**	**POS**	**ISF**	***P*-value**
Bacteroidetes	47.9 ± 3.54	49.9 ± 1.96	48.1 ± 3.40	0.791
Deferribacteres	0.93 ± 0.57	0.76 ± 0.46	0.82 ± 0.42	0.822
Epsilonbacteraeota	9.93 ± 2.74	6.52 ± 1.42	7.20 ± 2.14	0.628
Firmicutes	34.1 ± 3.64	35.7 ± 1.66	34.3 ± 3.08	0.793
Fusobacteria	1.04 ± 0.48	1.45 ± 1.06	1.81 ± 0.85	0.918
Kiritimatiellaeota	0.47 ± 0.47	0.02 ± 0.01	0.07 ± 0.04	0.696
Proteobacteria	3.66 ± 0.86	2.30 ± 0.33	4.39 ± 1.11	0.198
Spirochaetes	1.42 ± 0.38	2.58 ± 0.63	2.80 ± 0.99	0.561

[1]Abbreviations: DPI: days post-inoculation; PRRSV: porcine reproductive and respiratory syndrome virus; ISF = isoflavones; NEG: Control diet + uninfected; POS: Control diet + PRRSV-infection; ISF: Control diet + ISF + PRRSV-infection.
[2]Data are expressed as least-square mean ± standard error of the mean (SEM) for each treatment group.
[3]Only phyla where at least one mean was > 0.30% relative abundance were considered for interpretation.

Table 5.3. Relative abundance of bacterial genera detected in fecal samples at -2 DPI[1,2]

	Treatment			
Bacterial genus by phyla[3]	**NEG**	**POS**	**ISF**	***P*-value**
Bacteroidetes				
Alloprevotella	6.55 ± 1.75	8.05 ± 1.58	6.14 ± 1.69	0.606
Bacteroides	0.84 ± 0.47	0.97 ± 0.33	1.60 ± 0.55	0.581
dgA-11 gut group	0.80 ± 0.36	0.54 ± 0.14	1.16 ± 0.32	0.310
(F) Muribaculaceae Porphyromonadaceae Unassigned	2.71 ± 0.61	4.59 ± 1.39	7.90 ± 2.49	0.354
(F) Muribaculaceae Unassigned	2.20 ± 0.64	1.51 ± 0.29	1.46 ± 0.32	0.493
Parabacteroides	1.49 ± 0.27	1.38 ± 0.26	1.53 ± 0.28	0.961
Prevotella 1	1.48 ± 0.29	2.48 ± 0.42	2.32 ± 0.63	0.415
Prevotella 2	4.08 ± 0.84	3.22 ± 0.53	2.44 ± 0.60	0.192
Prevotella 9	13.9 ± 3.83	9.30 ± 1.36	7.16 ± 1.18	0.241
Prevotellaceae NK3B31 group	2.60 ± 0.52	2.40 ± 0.36	2.05 ± 0.32	0.614
Prevotellaceae UCG-003	3.70 ± 1.01	5.60 ± 0.80	3.92 ± 1.06	0.136
Rikenellaceae RC9 gut group	3.53 ± 0.39	4.94 ± 0.72	5.17 ± 0.87	0.340
Firmicutes				
Agathobacter	2.85 ± 1.18	2.17 ± 0.50	1.04 ± 0.29	0.108
Anaerovibrio	2.22 ± 0.92	1.19 ± 0.26	1.02 ± 0.49	0.242
(F) Eubacterium coprostanoligenes group	0.56 ± 0.10	1.17 ± 0.24	1.65 ± 0.64	0.343
Faecalibacterium	2.16 ± 0.70	1.79 ± 0.35	1.31 ± 0.23	0.548
Lachnospiraceae NK4A136 group	2.02 ± 0.63	2.67 ± 0.79	1.48 ± 0.62	0.349
(F) Lachnospiraceae Unassigned	2.72 ± 0.53	2.90 ± 0.45	2.20 ± 0.43	0.408
Lactobacillus	4.37 ± 1.81	3.81 ± 0.90	3.39 ± 1.20	0.644
Megasphaera	2.69 ± 2.10	0.19 ± 0.13	2.94 ± 2.12	0.196
Phascolarctobacterium	1.15 ± 0.35	1.79 ± 0.34	1.63 ± 0.40	0.532
Roseburia	1.35 ± 0.41[b]	0.59 ± 0.11[a]	0.58 ± 0.15[a]	0.039
Ruminococcaceae UCG-002	1.05 ± 0.25	1.74 ± 0.32	1.95 ± 0.47	0.411
(F) Veillonellaceae Unassigned	0.54 ± 0.25	1.83 ± 0.73	1.87 ± 1.23	0.665
Fusobacteria				
Fusobacterium	1.04 ± 0.48	1.45 ± 1.06	1.81 ± 0.85	0.910
Proteobacteria				
Campylobacter	9.48 ± 2.64	6.30 ± 1.43	6.00 ± 1.88	0.546
Desulfovibrio	0.84 ± 0.14	1.10 ± 0.15	1.09 ± 0.23	0.645
Helicobacter	0.46 ± 0.18	0.23 ± 0.11	1.21 ± 0.77	0.324
Succinivibrio	1.07 ± 0.59	0.40 ± 0.22	2.05 ± 0.84	0.109
Spirochaetes				
Treponema 2	1.30 ± 0.35	2.51 ± 0.63	2.66 ± 0.99	0.568

[ab]Means without a common superscript letter differ ($P < 0.05$).

[1]Abbreviations: DPI: days post-inoculation; PRRSV: porcine reproductive and respiratory syndrome virus; ISF = isoflavones; NEG: Control diet + uninfected; POS: Control diet + PRRSV-infection; ISF: Control diet + ISF + PRRSV-infection.

[2]Data are expressed as least-square mean ± standard error of the mean (SEM) for each treatment group.

[3]Only genera with at least one mean with > 1.0% relative abundance were considered for interpretation. Bacterial genera names preceded by (F) indicates that genera are associated with a specific taxonomic family and has not yet been further classified.

Table 5.4. Percent relative abundance of bacterial phyla detected in fecal samples at 10 DPI[1,2]

	Treatment			
Bacterial phyla[3]	**NEG**	**POS**	**ISF**	***P*-value**
Bacteroidetes	50.1 ± 2.32	46.4 ± 2.7	42.4 ± 2.64	0.123
Elusimicrobia	0.41 ± 0.40	0.16 ± 0.10	0.09 ± 0.05	0.934
Epsilonbacteraeota	1.65 ± 0.42	3.62 ± 1.38	2.48 ± 0.72	0.536
Firmicutes	41.1 ± 2.76	36.8 ± 2.25	38.7 ± 3.00	0.533
Proteobacteria	5.15 ± 1.23[b]	10.4 ± 2.22[ab]	10.7 ± 1.71[a]	0.045
Spirochaetes	1.07 ± 0.48	1.72 ± 0.47	4.66 ± 1.57	0.070

[ab]Means without a common superscript letter differ ($P < 0.05$).

[1]Abbreviations: DPI: days post-inoculation; PRRSV: porcine reproductive and respiratory syndrome virus; ISF = isoflavones; NEG: Control diet + uninfected; POS: Control diet + PRRSV-infection; ISF: Control diet + ISF + PRRSV-infection.

[2]Data are expressed as least-square mean ± standard error of the mean (SEM) for each treatment group.

[3]Only phyla with >0.30 relative abundance were considered for interpretation.

Table 5.5. Relative abundance of bacterial genera detected in fecal samples at 10 DPI[1,2]

Bacterial genus by phyla[3]	Treatment			*P*-value
	NEG	POS	ISF	
Bacteroidetes				
Alloprevotella	4.46 ± 0.77	4.18 ± 0.81	3.32 ± 0.87	0.399
dgA-11 gut group	0.58 ± 0.30	0.79 ± 0.22	1.07 ± 0.46	0.563
(F) Muribaculaceae Porphyromonadaceae Unassigned	2.46 ± 0.39	5.98 ± 3.15	3.31 ± 0.73	0.593
(F) Muribaculaceae Unassigned	0.53 ± 0.16	1.08 ± 0.35	0.65 ± 0.15	0.243
Prevotella 1	2.52 ± 1.20	2.26 ± 0.57	2.52 ± 0.43	0.914
Prevotella 2	2.80 ± 0.32	2.30 ± 0.39	1.78 ± 0.21	0.100
Prevotella 9	18.4 ± 3.19[a]	9.56 ± 1.75[b]	7.63 ± 1.15[b]	0.002
Prevotellaceae NK3B31 group	1.71 ± 0.35[b]	3.64 ± 0.63[ab]	3.97 ± 0.61[a]	0.040
Prevotellaceae UCG-003	3.31 ± 0.91	4.28 ± 0.72	4.97 ± 0.86	0.408
(F) Prevotellaceae Unassigned	2.68 ± 0.37[a]	1.71 ± 0.28[a]	0.79 ± 0.15[b]	< 0.0001
Rikenellaceae RC9 gut group	7.25 ± 1.10	7.09 ± 2.72	7.79 ± 1.86	0.804
Firmicutes				
Acidaminococcus	1.14 ± 0.39	0.69 ± 0.27	1.49 ± 0.83	0.723
Agathobacter	1.48 ± 0.32	3.55 ± 1.75	1.78 ± 0.51	0.574
Anaerovibrio	1.25 ± 0.27[a]	0.65 ± 0.21[ab]	0.42 ± 0.14[b]	0.011
Dialister	2.83 ± 0.90[a]	1.40 ± 0.40[ab]	0.52 ± 0.13[b]	0.011
Faecalibacterium	1.53 ± 0.50	0.76 ± 0.18	0.62 ± 0.10	0.099
Lachnospiraceae NK4A136 group	1.23 ± 0.22	3.15 ± 0.88	2.82 ± 0.58	0.111
(F) Lachnospiraceae Unassigned	1.91 ± 0.48	2.37 ± 0.69	2.67 ± 1.07	0.869
Lactobacillus	5.72 ± 1.48	3.42 ± 0.65	3.78 ± 0.74	0.604
Megasphaera	4.14 ± 1.19	5.85 ± 1.85	5.21 ± 1.94	0.865
Roseburia	0.41 ± 0.16	0.78 ± 0.27	1.38 ± 0.59	0.246
Ruminococcaceae UCG-002	2.71 ± 0.37	1.51 ± 0.21	2.31 ± 0.51	0.173
(F) Veillonellaceae Unassigned	2.05 ± 0.45	1.43 ± 0.47	1.94 ± 0.44	0.319
Proteobacteria				
Campylobacter	1.58 ± 0.39	3.52 ± 1.37	2.31 ± 0.72	0.546
Succinivibrio	3.28 ± 1.27	7.81 ± 2.25	7.82 ± 1.88	0.155
Spirochaetes				
Treponema 2	1.04 ± 0.48	1.67 ± 0.46	4.64 ± 1.57	0.060

[ab]Means without a common superscript letter differ ($P < 0.05$).

[1]Abbreviations: DPI: days post-inoculation; PRRSV: porcine reproductive and respiratory syndrome virus; ISF = isoflavones; NEG: Control diet + uninfected; POS: Control diet + PRRSV-infection; ISF: Control diet + ISF + PRRSV-infection.

[2]Data are expressed as least-square mean ± standard error of the mean (SEM) for each treatment group.

[3]Only genera with at least one mean with > 1.0% relative abundance were considered for interpretation. Bacterial genera names preceded by (F) indicates that genera are associated with a specific taxonomic family and has not yet been further classified.

Table 5.6. Percent relative abundance of bacterial phyla detected in fecal samples at 144 DPI[1,2]

	Treatment			
Bacterial phyla[3]	**NEG**	**POS**	**ISF**	***P*-value**
Actinobacteria	0.43 ± 0.11[a]	0.20 ± 0.03[b]	0.24 ± 0.05[ab]	0.049
Bacteroidetes	30.6 ± 1.88	29.2 ± 1.09	29.3 ± 1.30	0.787
Elusimicrobia	0.05 ± 0.02	0.20 ± 0.05	0.49 ± 0.23	0.109
Firmicutes	30.1 ± 2.37	27.9 ± 1.27	30.1 ± 1.67	0.593
Kiritimatiellaeota	0.86 ± 0.18	1.33 ± 0.19	1.26 ± 0.18	0.129
Proteobacteria	25.3 ± 2.73	23.7 ± 2.44	18.7 ± 1.96	0.185
Spirochaetes	12.2 ± 2.69	16.9 ± 2.79	19.0 ± 3.14	0.346

[ab]Means without a common superscript letter differ ($P < 0.05$).
[1]Abbreviations: DPI: days post-inoculation; PRRSV: porcine reproductive and respiratory syndrome virus; ISF = isoflavones; NEG: Control diet + uninfected; POS: Control diet + PRRSV-infection; ISF: Control diet + ISF + PRRSV-infection.
[2]Data are expressed as least-square mean ± standard error of the mean (SEM) for each treatment group.
[3]Only phyla with at least one mean with >0.30% relative abundance were considered for interpretation.

Table 5.7. Relative abundance of bacterial genera detected in fecal samples at 144 DPI[1,2]

	Treatment			
Bacterial genus by phyla[3]	**NEG**	**POS**	**ISF**	***P*-value**
Bacteroidetes				
Bacteroides	1.09 ± 0.21	0.79 ± 0.17	0.99 ± 0.21	0.445
CAG-873	2.98 ± 1.49[a]	0.33 ± 0.12[b]	0.16 ± 0.05[b]	< 0.0001
dgA-11 gut group	0.88 ± 0.26	1.29 ± 0.45	1.53 ± 0.44	0.576
(F) Muribaculaceae Porphyromonadaceae Unassigned	1.30 ± 0.40	1.38 ± 0.38	1.12 ± 0.30	0.871
(F) Muribaculaceae Unassigned	3.20 ± 0.40	2.93 ± 0.66	2.41 ± 0.37	0.372
Parabacteroides	1.10 ± 0.15	0.75 ± 0.09	1.16 ± 0.16	0.149
Prevotella 1	0.68 ± 0.15	1.09 ± 0.19	1.59 ± 0.53	0.141
Prevotellaceae NK3B31 group	5.63 ± 1.19	5.66 ± 0.54	6.66 ± 0.82	0.599
Prevotellaceae UCG-001	3.35 ± 0.63	5.44 ± 0.94	4.24 ± 0.64	0.306
Prevotellaceae UCG-004	1.13 ± 0.22[a]	0.59 ± 0.05[b]	0.55 ± 0.06[b]	0.002
Rikenellaceae RC9 gut group	4.70 ± 0.47	4.33 ± 0.73	3.57 ± 0.42	0.337
Firmicutes				
Christensenellaceae R-7 group	3.43 ± 0.83	2.62 ± 0.50	2.10 ± 0.27	0.363
(F) Christensenellaceae Unassigned	0.79 ± 0.27	1.87 ± 0.31	2.15 ± 0.61	0.085
Clostridium sensu stricto 1	1.26 ± 0.24[a]	1.15 ± 0.21[a]	0.64 ± 0.09[b]	0.038
Family XIII AD3011 group	1.03 ± 0.18	1.63 ± 0.44	1.55 ± 0.28	0.471
Lachnospiraceae NK4A136 group	2.59 ± 0.64	1.75 ± 0.46	1.35 ± 0.30	0.155
(F) Lachnospiraceae Unassigned	1.60 ± 0.32[b]	2.87 ± 0.51[ab]	3.17 ± 0.50[a]	0.044
Lachnospiraceae XPB1014 group	2.07 ± 0.42[a]	1.05 ± 0.23[b]	1.12 ± 0.22[ab]	0.039
Ruminococcaceae NK4A214 group	2.19 ± 0.23	2.08 ± 0.31	1.78 ± 0.15	0.470
Ruminococcaceae UCG-002	1.54 ± 0.31[a]	0.83 ± 0.15[b]	0.92 ± 0.12[ab]	0.039
Ruminococcaceae UCG-005	2.09 ± 0.40	1.49 ± 0.20	1.58 ± 0.17	0.266
Ruminococcaceae UCG-014	0.45 ± 0.10[b]	0.79 ± 0.11[ab]	1.12 ± 0.25[a]	0.043
Ruminococcus 1	1.21 ± 0.36	1.01 ± 0.12	1.21 ± 0.17	0.782
(F) Veillonellaceae Unassigned	1.93 ± 0.64	1.07 ± 0.28	1.93 ± 0.45	0.170
Proteobacteria				
Succinivibrio	21.8 ± 3.06	18.3 ± 2.26	12.7 ± 1.99	0.060
Succinivibrionaceae UCG-001	2.77 ± 1.68	4.40 ± 1.35	4.20 ± 1.05	0.481
Spirochaetes				
Treponema 2	12.2 ± 2.68	16.9 ± 2.78	19.0 ± 3.14	0.348

[1]Abbreviations: DPI: days post-inoculation; PRRSV: porcine reproductive and respiratory syndrome virus; ISF = isoflavones; NEG: Control diet + uninfected; POS: Control diet + PRRSV-infection; ISF: Control diet + ISF + PRRSV-infection.

[2]Data are expressed as least-square mean ± standard error of the mean (SEM) for each treatment group.

[3]Only genera with at least one mean with > 1.0% relative abundance were considered for interpretation. Bacterial genera names preceded by (F) indicates that genera are associated with a specific taxonomic family and has not yet been further classified.

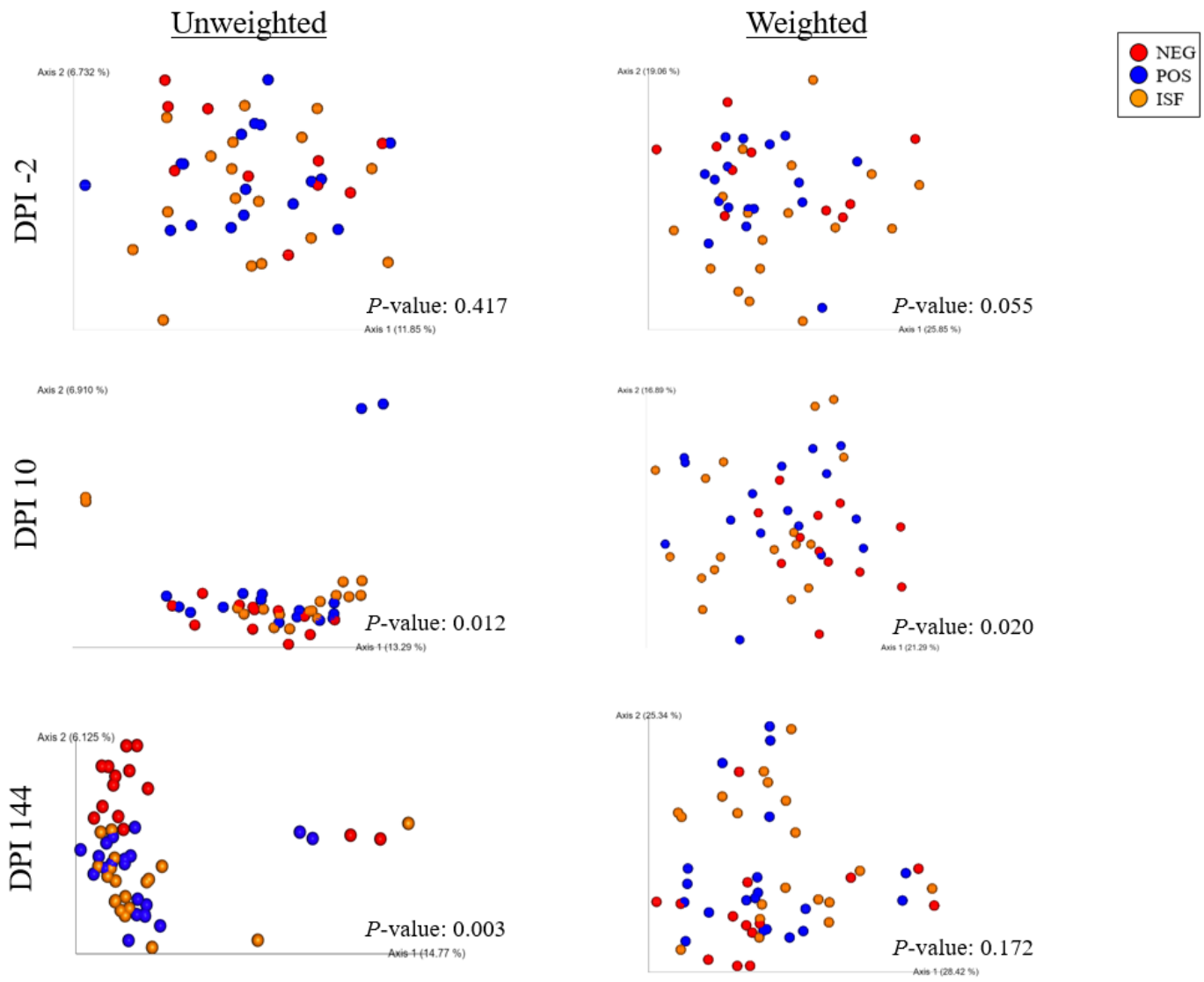

Figure 5.1. Principal co-ordinate analysis of unweighted and weighted UniFrac distances generated from fecal samples collected from individual pigs (*n* = 12-18 per treatment) at -2, 10, and 144 days post-inoculation. Treatment ID: NEG, control diet + uninfected; POS, control diet + PRRSV-infection; ISF, control diet + ISF + PRRSV-infection. Abbreviations: DPI, days post-inoculation; PRRSV, porcine reproductive and respiratory syndrome virus; ISF, isoflavones.

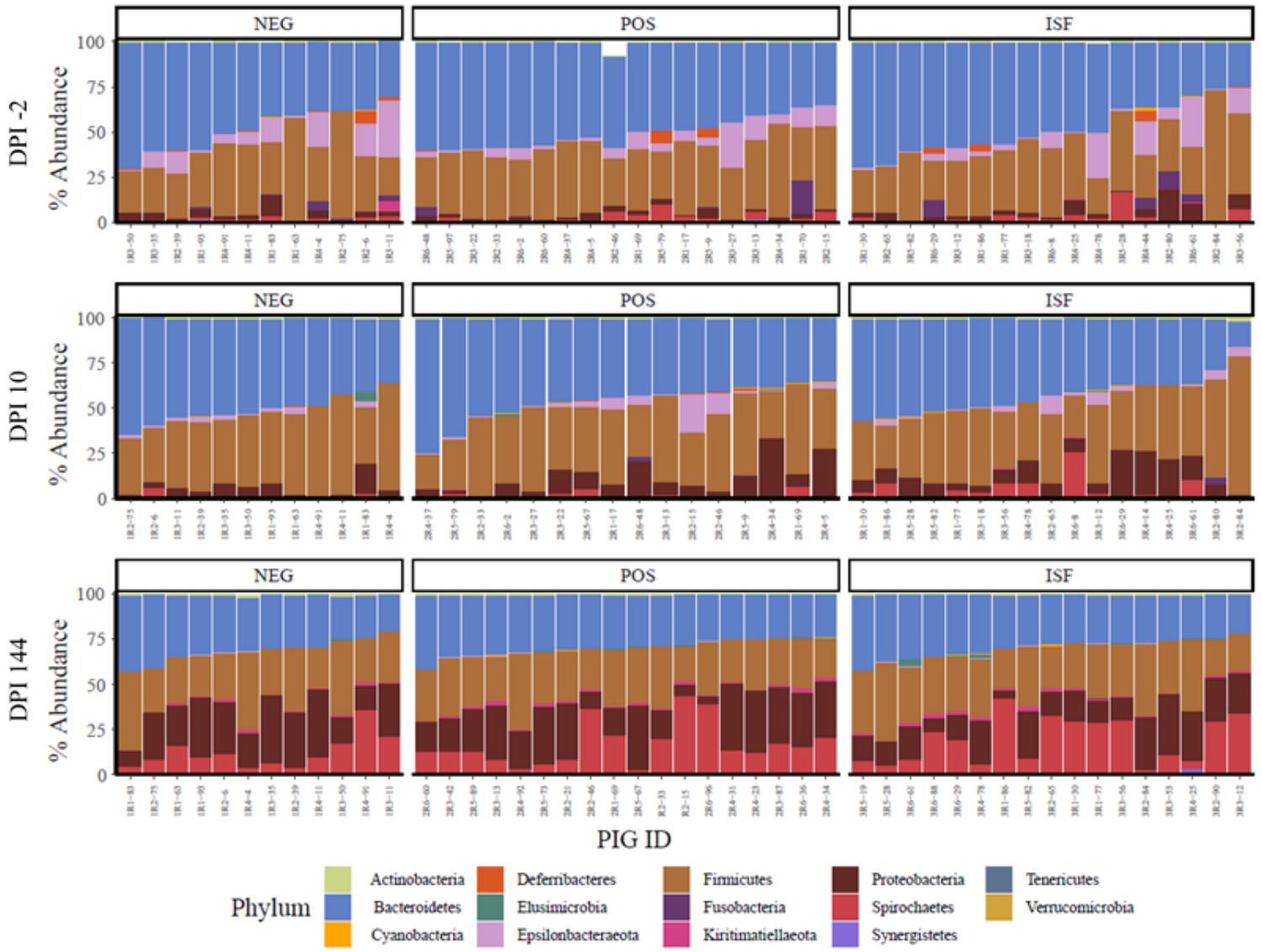

Figure 5.2. Percent relative abundance of bacterial phyla detected in fecal samples collected from individual pigs (*n* = 12-18 per treatment) at -2, 10, and 144 days post-inoculation. Treatment ID: NEG, control diet + uninfected; POS, control diet + PRRSV-infection; ISF, control diet + ISF + PRRSV-infection. Abbreviations: DPI, days post-inoculation; PRRSV, porcine reproductive and respiratory syndrome virus; ISF, isoflavones.

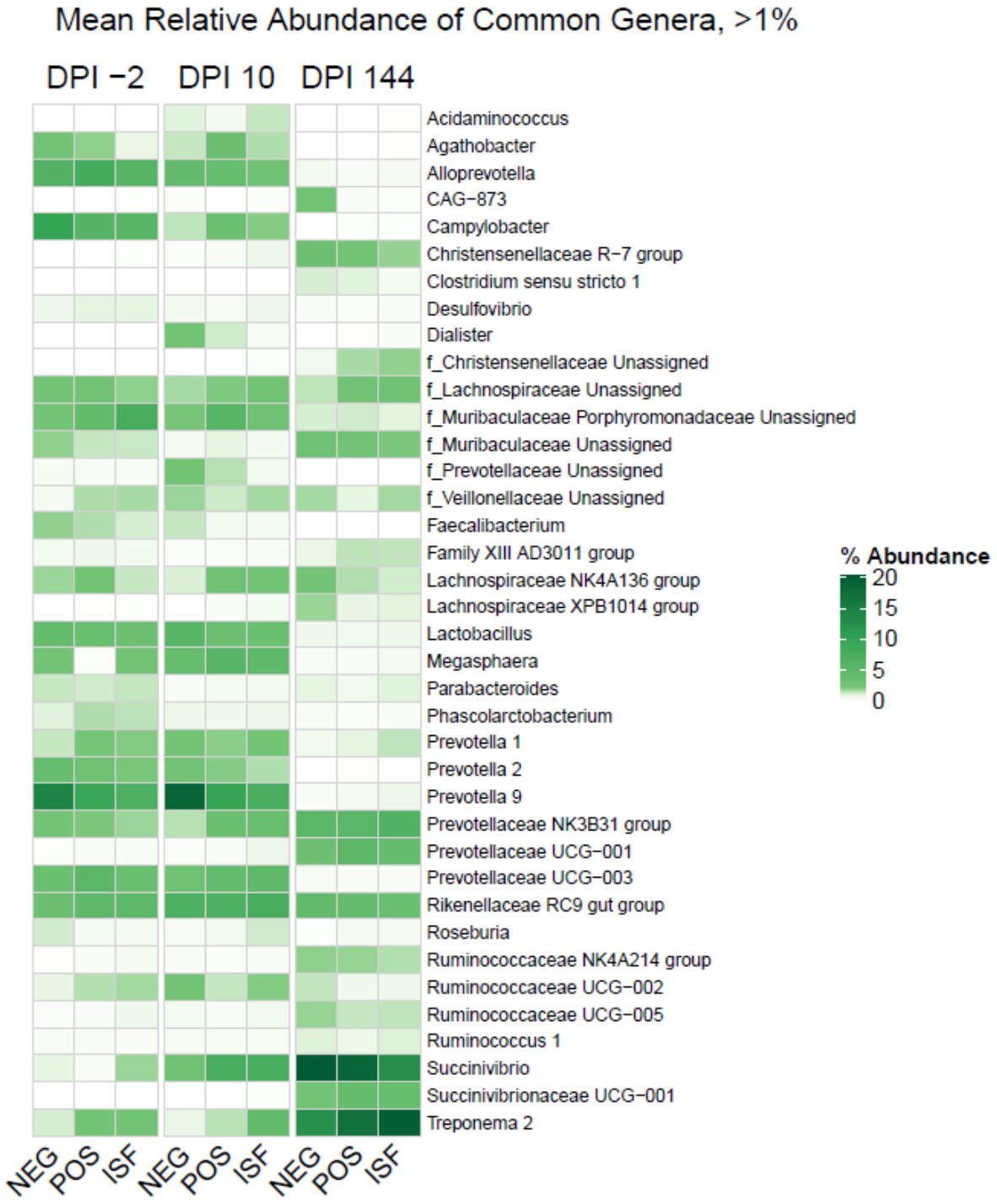

Figure 5.3. Heat map depicting differences in relative abundance of common genera (> 1.0% relative abundance) between experimental treatments at -2, 10, and 144 days post-inoculation. Treatment ID: NEG, control diet + uninfected; POS, control diet + PRRSV-infection; ISF, control diet + ISF + PRRSV-infection. Abbreviations: DPI, days post-inoculation; PRRSV, porcine reproductive and respiratory syndrome virus; ISF, isoflavones.

Figure 5.4. Heat map depicting differences in relative abundance of rare genera (< 1.0% relative abundance) between experimental treatments at -2, 10, and 144 days post-inoculation. Treatment ID: NEG, control diet + uninfected; POS, control diet + PRRSV-infection; ISF, control diet + ISF + PRRSV-infection. Abbreviations: DPI, days post-inoculation; PRRSV, porcine reproductive and respiratory syndrome virus; ISF, isoflavones.

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CHAPTER 6: GENERAL CONCLUSIONS

The overall focus of this dissertation was to determine if dietary soy isoflavones provide performance benefits to growing pigs faced with a pathogenic challenge and identify potential biological mechanisms. Three experimental aims and objectives were addressed, and their conclusions are summarized below.

Regarding the first experiment, intervention of an acute porcine reproductive and respiratory syndrome viral (**PRRSV**) infection in weanling pigs resulted in only minor changes in performance by industry standard measures. The influence of isoflavones on growth of infected pigs was mild and less pronounced than previous PRRSV-infection models evaluating individual isoflavone supplementation or soy protein inclusion. However, there were significant differences in a few of the cellular immune responses, which indicated that isoflavones may influence systemic immune responses in the context of this challenge model. The key differences noted were reduced band neutrophilia during the early acute infection period and an increased helper-to-cytotoxic T-cell ratio during the mid-to-late acute infection period. Both findings suggest a more appropriate immune response in the face of pathogenic challenge, though these effects may not be specific to PRRSV. Due to decreased mobilization of immature innate immune cells and increased proportions of helper T-cells, we speculate that isoflavones may be providing support to the immune system in its response to PRRSV but due to the short timeline utilized in this experiment we may have missed the benefits of that support as pigs transitioned from acute infection to recovery.

In order to address this speculation, a second experiment was performed using the same PRRSV-challenge protocol. However, this experiment was conducted following an extended timeline to capture the transition from acute infection to recovery in order to determine what

influence isoflavones had on performance over the entire growth period from weaning to market. Similar to the first experiment, isoflavones had inconsistent effects on growth performance across the entire growth period, resulting mainly in lower finishing weights in supplemented pigs, a finding that was not maintained when pigs reached market for processing. Again, as in the first experiment, isoflavones appeared to influence certain cellular immune responses but not in the same way. Instead of decreasing band neutrophil proportions, isoflavones in this experiment increased the relative proportion and count of mature neutrophils during the acute infection period and increased the proportion of memory T-cells at 20 days post-inoculation (**DPI**), but had no influence on other T-cell subpopulations. These effects may have influenced the differences observed between experimental treatments during the recovery period, including decreased time to total oral fluid viral clearance and development of anti-PRRSV neutralizing antibodies in isoflavone-supplemented pigs, but the connection remains unclear. However, one particularly striking outcome from this study was the impact isoflavones had on total pathogen-associated mortality. Recognizing that this model was complicated by the presence of secondary bacterial infections, isoflavone supplementation decreased mortality in supplemented pigs by ~50% compared with those not receiving isoflavones. If a true finding, this reduction of pig losses has significant financial implications for pork producers facing PRRSV and associated secondary infections in their systems.

Similar to the unclear connection between changes in immune outcomes and downstream effects on recovery outcomes, an additional source of uncertainty regarding the biological mechanisms of isoflavone activity *in vivo* is whether isoflavones induce a majority of their activity directly or indirectly. Since they are a dietary component, they are subject to metabolism by both the animal and the gastrointestinal microbiome. To address this question, a third and

final experiment was performed to determine the influence of dietary isoflavones on the longitudinal composition of the gastrointestinal microbiome. Findings from this experiment suggested there were minimal effects on the global diversity of the fecal microbiome by dietary isoflavones with a majority of differences appearing to be related to PRRSV-infection directly. This is not unexpected regarding the impact of PRRSV, which has been shown to decrease microbiome diversity in pigs across several independent studies. However, differences due to isoflavone supplementation suggest that isoflavones only exert weak influence on the composition of microbial communities in the colon. Thus, our results suggest that positive effects of isoflavones observed in our experiments are due to direct action of isoflavones and not by modulation of the microbiome or its metabolic activity.

Considering the collective outcomes of these experiments, it is reasonable to conclude that soy isoflavones do not negatively impact the productivity of pathogen-challenged pigs and may provide some level of immune system support. However, there are several limitations that must be considered as pertinent to our interpretations. Due to low animal numbers and limitations of facilities available to conduct disease research in live animal models, it may be difficult to apply these findings in a commercial context, particularly when interpreting mortality rates. Additionally, due to the immunosuppressive properties of PRRSV and differences in strain virulence factors, it can be very difficult to induce the same infection conditions in successive experiments. Not being able to fully control the presence of secondary, opportunistic infections that are common in pigs challenged with PRRSV decreases the likelihood of perfectly reproducing immune outcomes, the effect of which is compounded by low animal numbers. Moving forward, it will be critical to identify locations and opportunities to use more animals for

this infection model, specifically if a highly virulent PRRSV strain like the one utilized for these experiments is utilized.

Another limitation of this research was the static concentration of isoflavones utilized. While it allowed us to identify potential mechanisms of action for isoflavones when included at levels typical of commercial swine diets, use of a static dietary concentration does not address whether increasing levels in the diet would confer additional benefits. Since we can reasonably conclude that isoflavones do not negatively impact performance in pathogen challenged pigs, a logical next step is to evaluate if increasing isoflavones in the diet would additionally benefit pigs facing immune stress or if at a certain level they begin to decrease performance. For that question to be addressed, we first need to understand what true isoflavone concentrations are observed across production phases in swine production systems at which point titration study approaches can be designed. Regardless of these limitations, based on our findings we suggest that soy isoflavones still exhibit potential to play a key part in the role of nutritional management of disease in modern livestock production and more research in this area is merited.

APPENDIX A: CONCENTRATIONS OF SOY ISOFLAVONES IN STANDARD SWINE DIETS ACROSS PRODUCTION PHASES - A PRELIMINARY SURVEY STUDY

Introduction

Looking to the future of soy isoflavone (**ISF**) research in swine production, a logical next step is to investigate the effect of increasing isoflavone concentrations in the diets fed to pigs facing a pathogenic challenge over that which is typically found in standard formulations. The experiments described in this dissertation utilized soy isoflavone concentrations that would mimic those expected to naturally occur in a corn-soybean meal (**SBM**) diet containing approximately 20% SBM. However, that inclusion rate represents an average value; in reality, the inclusion rate of SBM and utilization of further processed soy protein products varies by life stage in U.S. swine production. From weaning to market, crude protein (**CP**) content, which is mainly provided by soy protein sources in the United States, ranges from ~25% in young pigs (5-7 kg body weight, **BW**) to less than 15% in finishing pigs (≥ 100 kg) due to differences in protein requirements for growth. For that reason, it is pertinent to understand the average levels of ISF in standard swine diets across different life stages. The objective of this preliminary survey was to investigate and categorize soy ISF concentrations of standard swine diet formulations by production phase. As such, we sought to provide a starting point for future research opportunities, particularly when determining experimental soy ISF inclusion levels.

Materials and Methods

Sample Collection and Analysis

A total of 16 feed samples were collected: 8 from standard diet formulations fed to resident swine herds at the University of Illinois at Urbana-Champaign and 8 from diet formulations used at regional, commercial swine farms. University feed samples were collected

either on site at the University of Illinois Swine Research Center (**SRC**) (Urbana, IL) or directly following mixing at the University of Illinois feed mill (Urbana, IL). Commercial feed samples were collected directly following mixing at the Zimmerman Feed and Grain, Inc. feed mill (Forrest, IL). Formulations from which feed samples were collected were categorized by standard swine production phases for both locations. For the sake of this dissertation, please refer to **Table A.1** for specifications of individual swine production phases. Production phases represented by the university feed samples include: Gestation, Lactation, Nursery Phases 1-3, Grower Phases 1-2, and Finisher Phase. Production phases represented by commercial feed samples include: Early Nursery Phase (comparable to University Nursery Phase 1), Late Nursery Phase (comparable to University Nursery Phase 3), Grower Phase (comparable to University Grower Phases 1 and 2), and Late Finisher Phase.

For all diets sampled, soybean meal (**SBM**) was the primary source of soy protein and inclusion levels were recorded. Please note that the commercial feed samples were collected from diets that contained byproduct feed ingredients that may contain additional sources of soy protein that were not able to be quantified. For commercial diets, only four diet formulations were tested but two individual samples were taken from each formulation and analyzed separately. Samples were collected into plastic bags from which a random sub-sample ≤ 10 g in weight was prepared and submitted for ISF analysis. Isoflavone concentrations of individual diets were quantified via HPLC at the USDA-ARS National Center for Agricultural Utilization Research (Peoria, IL) according to procedures of Berhow et al. (2006).

Results

Results for SBM inclusion rates and isoflavone concentrations of individual diets can be found in **Table A.2**. The average SBM inclusion level of diet samples was approximately 21.5%

for university diets (range: 14% to 31.5%) and 19.5% for commercial diets (range: 6.4% to 29.6%). Highest inclusion levels were found in late-nursery phase formulations and lowest inclusion levels were found in finisher phase formulations for both university and commercial diets. The average total ISF content of diet samples was approximately 867 mg/kg for university diets (range: 563 ± 16.65 mg/kg to 1,080 ± 18.5 mg/kg) and 1,246 mg/kg for commercial diets (range: 535 ± 20.4 mg/kg to 2,055 ± 157 mg/kg). Though the average SBM inclusion levels across all production phases were relatively similar between university and commercial diets, the higher average ISF content of the commercial diets may be partially explained by the presence of additional soy protein present in byproduct feeds utilized in these diets that were not able to be quantified. Referencing back to research summarized in this dissertation, our experimental ISF inclusion levels of 1,500-1,600 mg/kg was greater than all of the university diet samples analyzed, but only four of the diet samples analyzed from our commercial source. This could suggest that utilization of byproduct feeds contribute to greater levels of ISF in swine diets, over that which is expected based on SBM inclusion rates alone. Within both university and commercial diets, average variability across analyzed replicates of diet samples from different production phases for individual and total ISF content was relatively low (CV range: 2.97% to 5.08%), though there were some samples that exhibited greater levels of variability particularly for genistein content (e.g., 10.6% CV for total genistein for University Nursery Phase 1 diet; 18.4% CV for total genistein for Commercial Mid-Late Nursery Phase Sample 1). However, more samples are needed to better understand variability across different diets, especially when considering differences in soy ingredient sources and types. Additional visualizations of these data can be found in **Figures A.1 and A.2**.

Tables and Figures

Table A.1. Swine production phase descriptions[1]

Production Phase	Description
Gestation	Includes pregnant female pigs from time of breeding/conception until farrowing (~115 days)
Lactation	Includes female pigs from farrowing until weaning (~21 days)
Nursery	Includes pigs from weaning until reaching ~25 kg body weight
Grower	Includes growing pigs weighing 25 kg-70 kg
Finisher	Includes growing pigs weighing 70 kg-130 kg

[1]Descriptions adapted from information gathered from Pork Checkoff (2016) and the American Association of Swine Veterinarians (2009).

Table A.2. Isoflavone and saponin content of swine diets categorized by production phase grouped by sampling location[1]

Diet ID	SBM Inclusion (%)	Isoflavones (mg/kg)[2]			
		Genistein	Daidzein	Glycitein	Total
University Diets					
Gestation	24.1	263 ± 2.15	214 ± 13.6	86.2 ± 2.77	564 ± 16.7
Lactation	11.6	475 ± 2.60	401 ± 4.27	113 ± 3.01	989 ± 9.49
Nursery Phase					
Phase 1	22.0	369 ± 39.2	294 ± 22.9	81.9 ± 3.46	745 ± 60.2
Phase 2	29.5	518 ± 1.80	430 ± 6.65	133 ± 10.7	1,081 ± 18.5
Phase 3	31.5	538 ± 29.1	420 ± 24.9	112 ± 6.91	1,069 ± 60.7
Grower Phase					
Phase 1	22.0	372 ± 35.8	326 ± 34.9	155 ± 12.5	854 ± 81.8
Phase 2	17.0	466 ± 22.4	357 ± 6.37	108 ± 2.37	931 ± 26.7
Finisher Phase	14.0	321 ± 20.5	246 ± 5.78	135 ± 5.30	703 ± 30.8
Commercial Diets[3]					
Mid/Late Nursery Phase[4]					
Sample 1	27.9	652 ± 21.5	1,073 ± 28.5	430 ± 6.65	1,967 ± 59.1
Sample 2	27.9	765 ± 141	1,069 ± 38.5	223 ± 7.31	2,056 ± 157
Late Nursery Phase					
Sample 1	29.6	684 ± 12.3	627 ± 10.4	222 ± 3.28	1,533 ± 25.6
Sample 2	29.6	707 ± 25.8	640 ± 20.8	224 ± 6.80	1,570 ± 52.3
Early Finisher Phase					
Sample 1	14.4	407 ± 25.4	377 ± 3.88	126 ± 2.81	911 ± 26.5
Sample 2	14.4	361 ± 4.00	368 ± 9.37	124 ± 7.58	853 ± 20.5
Late Finisher Phase					
Sample 1	6.4	194 ± 8.84	278 ± 9.20	71.1 ± 3.76	543 ± 20.9
Sample 2	6.4	191 ± 3.01	270 ± 15.3	75.4 ± 2.39	536 ± 20.4

[1]Abbreviations: SBM, soybean meal

[2] Data are expressed as average concentration ± standard deviation for each diet sample.

[3]Commercial feed samples were collected from diets that contain byproduct feed ingredients that may contain additional sources of soy protein that were not able to be quantified; only SBM reported.

[4]Diet also contained 1.3% Soycomil-P (ADM, Decatur, IL), a low anti-nutritional factor soy protein product.

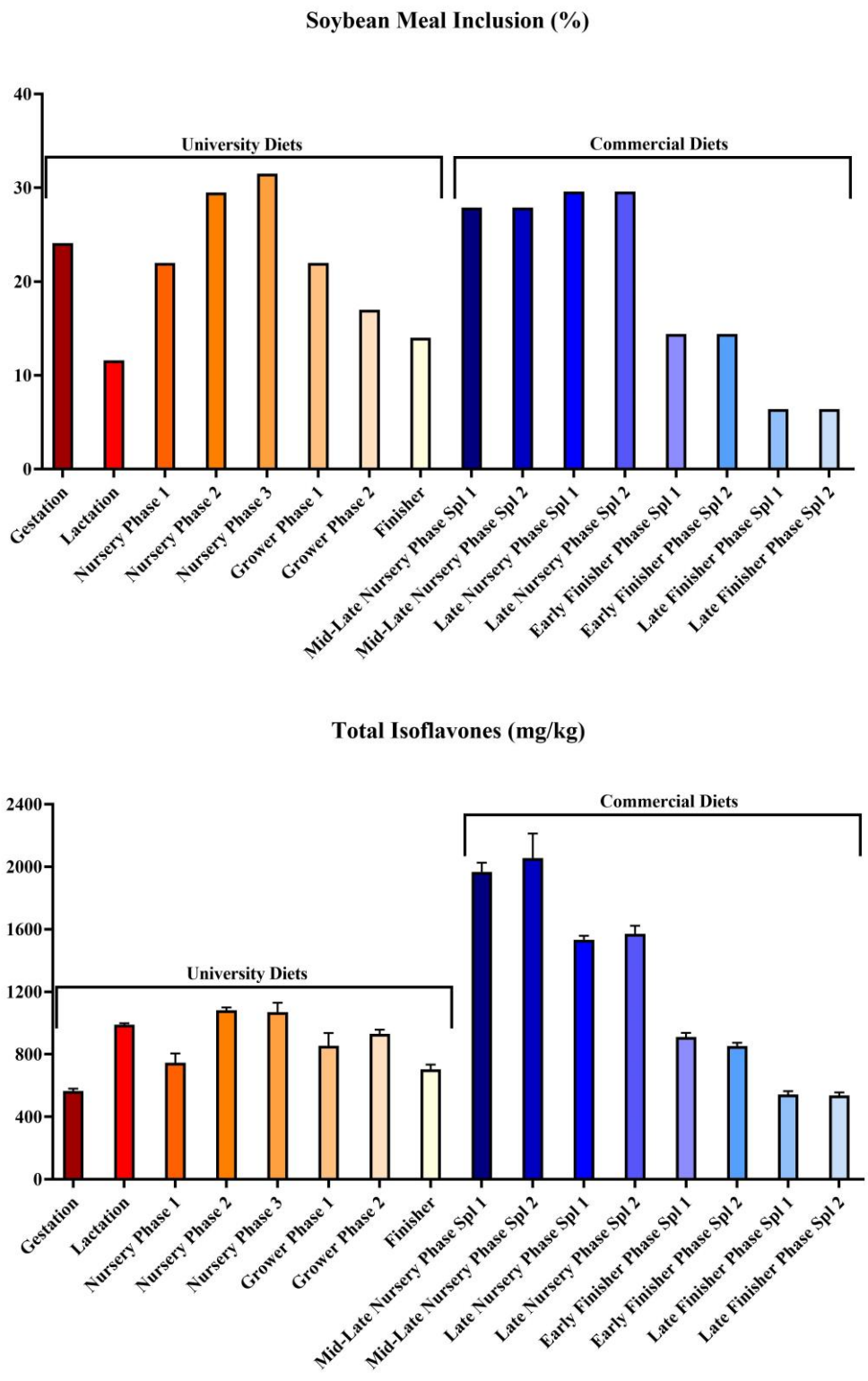
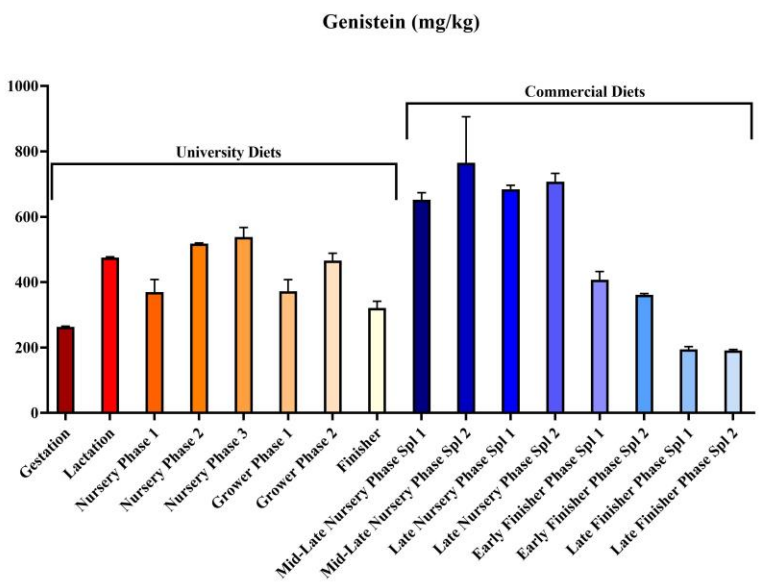
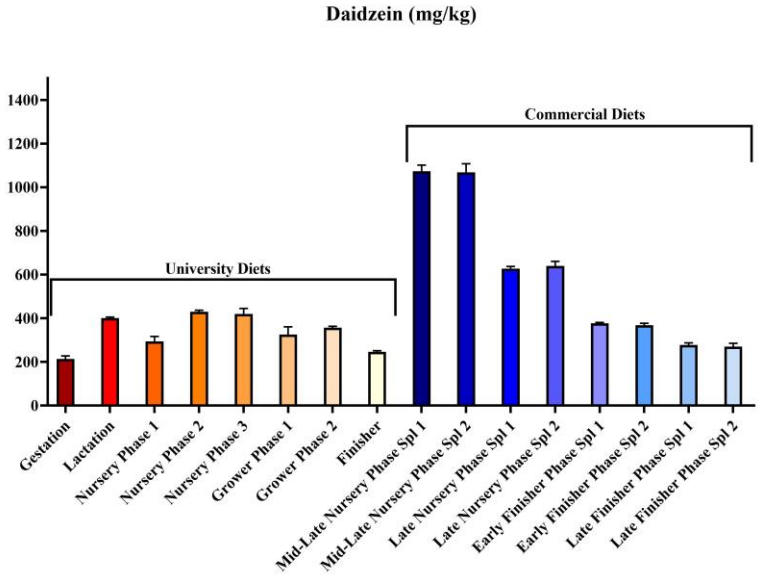


Figure A.1. Total dietary soybean meal inclusion (%) and total analyzed isoflavone concentrations (mg/kg) by diet sample, categorized by diet source (University vs. Commercial): Abbreviations: Spl, sample.





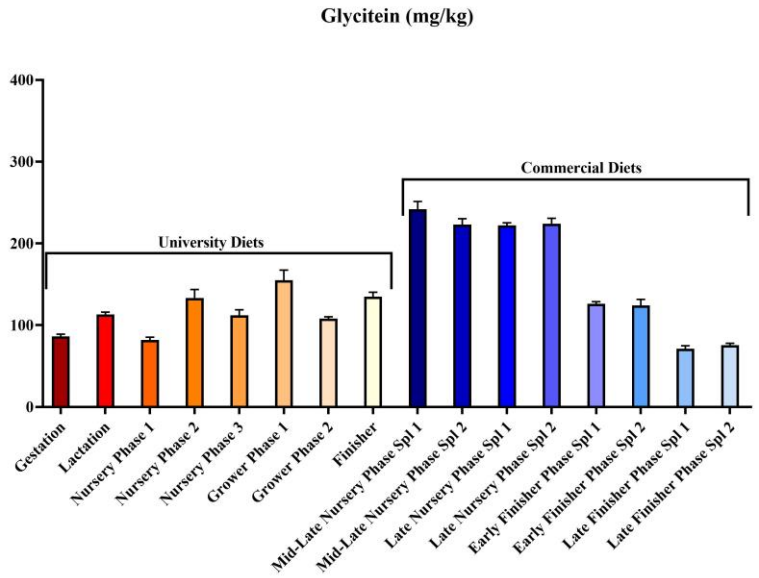


Figure A.2. Analyzed genistein, daidzein, and glycitein concentrations (mg/kg) by diet sample, categorized by diet source (University vs. Commercial). Abbreviations: Spl, sample.

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APPENDIX B: DUNNETT'S ADJUSTMENT OF IMMUNE OUTCOME DATA FROM CHAPTER 3

Introduction

By request of the committee following my preliminary examination, in order to determine if dietary treatment modulated the immune response to PRRSV-infection in such a way that immune parameters were returned to or maintained at values comparable to the uninfected control group, certain data from this experiment were reanalyzed and a Dunnett's adjustment was performed. Please note that in our original approach, we performed two separate statistical comparisons: 1) between our uninfected control group vs. our positive control group and 2) between all of our infected groups; this is the first time we compared our uninfected control against all infected treatments. These data correspond to those described in Chapter 3 of this dissertation document.

Materials and Methods

Experimental Outcomes Reanalyzed

The following immune outcomes were able to be reanalyzed for a Dunnett's adjustment. Non-immune outcomes and outcomes for which no values were available for the uninfected control group were omitted:

- Rectal Temperatures
- RBC Measures
- WBC Measures
- Serum Cytokines (TNF-α and IFN-α only)
- Immunophenotyping Measures

Statistical Analysis

For this reanalysis, a 1-way ANOVA was conducted using the MIXED procedure of SAS 9.4 (SAS Institute, Inc., Cary, NC) to assess the overall effect of treatment separated by DPI for repeated measures and utilizing Rep as a random variable. As a reminder, each treatment represented a single level of soy protein (SPC/Control vs. ETSBM), a single level of ISF supplementation (no ISF added vs. ISF added), and a single level of infection (uninfected vs. PRRSV-infected), which resulted in 5 experimental treatments (see **Table B.1** below). Significance was accepted at $P \leq 0.05$.

Results

Please see **Tables B.2-B.6** below.

Tables

Table B.1. Experimental treatments[1]

Treatment	Dietary treatment	Infection status[2]
Control	Soy protein concentrate	Uninfected
Control	Soy protein concentrate	PRRSV-infected
Control + ISF	Soy protein concentrate + ISF	PRRSV-infected
ETSBM[3]	ETSBM	PRRSV-infected
ETSBM[3] + ISF	ETSBM + ISF	PRRSV-infected

[1]Abbreviations: ISF, soy isoflavones; PRRSV, porcine reproductive and respiratory syndrome virus, Mh, *Mycoplasma hyopneumoniae*.
[2]All pigs were naturally co-infected with Mh prior to the start of the study at the source farm.
[3]Enzyme-treated soybean meal (ETSBM) manufactured to contain a gentle soya-yeast (10% yeast components) supplement for piglet feed with a low content of anti-nutritional factors (trypsin inhibitors, antigens, and flatulent oligosaccharides); Hamlet Protein, Findlay, OH.

Table B.2. Effects of dietary soy isoflavones level and porcine reproductive and respiratory virus (PRRSV) infection on daily rectal temperatures (ºC) of weanling pigs[1]

	Uninfected	PRRSV-infected					*P*-value
DPI	Control[2]	Control[2]	Control + ISF[2]	ETSBM[2]	ETSBM + ISF[2]	SEM	Treatment
0	39.36	39.86[†]	39.91[†]	39.69	39.65	0.25	0.043
3	39.48	40.07	39.92	40.35[†]	40.11[†]	0.24	0.019
6	39.65	40.12	40.42[†]	40.47[†]	40.18[†]	0.46	0.038
8	39.58	40.36	40.69	40.50	40.62	0.25	0.0004
12	40.01	40.24	40.68	40.50	40.49	0.18	0.076
14	39.88	40.29	40.21	40.29	40.40	0.18	0.479

[†]Difference ($P < 0.05$) between uninfected and infected groups by Dunnett's adjustment when overall treatment effect was present.

[1]Values represent least square means of 10 to 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI = day post-inoculation; ISF = isoflavones.

[2]The following diet names have been assigned to a respective experimental diet groups: *Control*: soy protein concentrate + no supplemented ISF, fed to both uninfected and PRRSV-infected control pigs; *Control + ISF*: soy protein concentrate + supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM*: ETSBM + no supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM + ISF*: ETSBM + supplemented ISF, fed to PRRSV-infected pigs only.

Table B.3. Effects of dietary soy isoflavone levels and porcine reproductive and respiratory virus (PRRSV) infection on red blood cell measures in weanling pigs[1]

	Uninfected	PRRSV-infected					*P*-value
Item	Control[2]	Control[2]	Control + ISF[2]	ETSBM[2]	ETSBM + ISF[2]	SEM	Treatment
RBC, × 10^6/μL							
0 DPI	6.53	6.66	6.84	6.49	6.81	0.23	0.367
3 DPI	6.20	6.00	6.14	5.90	6.29	0.24	0.204
6 DPI	5.97	6.05	6.17	5.87	6.00	0.24	0.773
14 DPI	5.85	5.31	5.58	5.25	5.43	0.17	0.111
HGB, g/dL							
0 DPI	12.3	11.9	12.5	12.2	12.5	0.36	0.792
3 DPI	11.5	10.6	11.1	10.8	11.4	0.30	0.080
6 DPI	10.9	10.4	10.9	10.5	10.8	0.36	0.604
14 DPI	11.0	9.0†	9.5†	9.1†	9.5†	0.45	0.001
HCT, %							
0 DPI	35.1	37.4	39.1	37.9	39.1	1.80	0.513
3 DPI	35.7	33.4	34.9	33.8	35.6	0.93	0.064
6 DPI	34.0	32.9	34.4	33.1	33.7	1.00	0.666
14 DPI	34.7	28.5†	30.3†	28.8†	30.0†	1.36	0.001
MCV, fl							
0 DPI	58.6	56.2	57.2	58.5	57.5	2.02	0.584
3 DPI	57.9	55.7	56.9	57.3	56.7	1.46	0.515
6 DPI	57.4	54.6	55.8	56.4	56.1	1.47	0.379
14 DPI	59.6	53.7†	54.2†	55.0†	55.3†	1.58	0.005
MCH, pg							
0 DPI	18.6	17.9	18.3	18.8	18.4	0.73	0.598
3 DPI	18.4	17.7	18.1	18.3	18.1	0.65	0.656
6 DPI	18.2	17.3	17.7	17.9	18.0	0.54	0.338
14 DPI	18.8	17.0†	17.1†	17.4†	17.5†	0.54	0.009
MCHC, g/dL							
0 DPI	31.8	31.6	32.0	32.0	32.0	0.26	0.851
3 DPI	31.7	31.6[b]	31.9[b]	31.9[b]	29.3[a]	1.25	0.472
6 DPI	31.7	31.5	31.8	31.6	32.0	0.32	0.573
14 DPI	31.5	31.6	31.6	31.6	31.7	0.24	0.996

†Difference ($P < 0.05$) between uninfected and infected groups by Dunnett's adjustment when overall treatment effect was present.

[1]Values represent least square means of 10 to 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI = day post-inoculation; ISF = isoflavones; RBC = red blood cells; HGB = hemoglobin; HCT = packed cell volume; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration

[2]The following diet names have been assigned to a respective experimental diet groups: *Control*: soy protein concentrate + no supplemented ISF, fed to both uninfected and PRRSV-infected control pigs; *Control + ISF*: soy protein concentrate + supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM*: ETSBM + no supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM + ISF*: ETSBM + supplemented ISF, fed to PRRSV-infected pigs only.

Table B.4. Effects of dietary soy isoflavones level and porcine reproductive and respiratory virus (PRRSV) infection on differential leukocyte counts and relative population proportions in weanling pigs[1]

	Uninfected	PRRSV-infected					*P*-value
Item	Control[2]	Control[2]	Control + ISF[2]	ETSBM[2]	ETSBM + ISF[2]	SEM	Treatment
WBC, × 10^3/µL							
0 DPI	15.2	13.3	13.4	14.8	17.2	1.89	0.270
3 DPI	16.1	12.2	9.83†	14.2	13.7	1.73	0.032
6 DPI	16.4	11.8	12.7	24.4	14.5	1.74	0.241
14 DPI	16.6	23.1	24.5	23.2	23.5	3.17	0.134
NEU, % of WBC							
0 DPI	49.1	45.3	38.9	47.5	40.5	3.09	0.091
3 DPI	46.3	56.5	57.1	57.8	55.1	5.83	0.637
6 DPI	38.7	51.5	54.2	59.1	47.4	5.10	0.059
14 DPI	35.7	49.9	43.2	46.9	39.4	4.82	0.197
BAND, % of WBC							
0 DPI	0.00	0.135	0.00	0.550	0.333	0.27	0.839
3 DPI	1.10	6.00	1.10	6.63	3.18	1.23	0.469
6 DPI	0.304	6.11†	2.45	5.25†	5.27†	1.64	0.011
14 DPI	0.087	1.27	4.44	1.77	3.63	1.17	0.119
LYM, % of WBC							
0 DPI	47.1	45.7	51.3	45.3	51.7	3.14	0.345
3 DPI	47.1	31.1	35.8	31.0	37.5	6.57	0.090
6 DPI	52.9	32.2†	32.8†	27.7†	38.1	4.81	0.007
14 DPI	51.7	39.5	41.9	45.0	47.7	4.37	0.359

†Difference ($P < 0.05$) between uninfected control and respective infected group by Dunnett's adjustment when overall treatment effect was present.

[1]Values represent least square means of 10 to 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI = day post-inoculation; ISF = isoflavones; WBC = white blood cells; NEU = neutrophils; BAND = immature neutrophils aka. band cells; LYM = lymphocytes; MONO = monocytes; EOSIN = eosinophils; BASO = basophils.

[2]The following diet names have been assigned to a respective experimental diet groups: *Control*: soy protein concentrate + no supplemented ISF, fed to both uninfected and PRRSV-infected control pigs; *Control + ISF*: soy protein concentrate + supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM*: ETSBM + no supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM + ISF*: ETSBM + supplemented ISF, fed to PRRSV-infected pigs only.

Table B.4. Continued

Item	Uninfected	PRRSV-infected					*P*-value
	Control[2]	Control[2]	Control + ISF[2]	ETSBM[2]	ETSBM + ISF[2]	SEM	Treatment
MONO, % of WBC							
0 DPI	2.77	7.09	7.19	5.56	6.00	1.23	0.131
3 DPI	4.47	3.25	2.10	2.98	2.62	0.98	0.395
6 DPI	6.06	6.50	7.53	5.42	5.62	1.24	0.705
14 DPI	4.73	4.81	5.86	3.70	5.07	1.47	0.847
EOSIN, % of WBC							
0 DPI	1.19	1.63	2.11	1.14	1.42	1.66	0.351
3 DPI	0.993	2.25	3.80[†]	1.04	1.31	1.22	0.014
6 DPI	1.79	3.06	2.49	2.25	3.40	0.92	0.769
14 DPI	2.95	4.07	4.15	2.19	3.61	1.22	0.394
BASO, % of WBC							
0 DPI	0.200	0.100	0.207	0.00	0.083	0.10	0.556
3 DPI	0.100	0.917	0.300	0.364	0.182	0.36	0.469
6 DPI	0.200	0.636	0.455	0.250	0.182	0.17	0.241
14 DPI	0.500	0.400	0.261	0.294	0.558	0.30	0.667

[†]Difference ($P < 0.05$) between uninfected control and respective infected group by Dunnett's adjustment when overall treatment effect was present.

[1]Values represent least square means of 10 to 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI = day post-inoculation; ISF = isoflavones; WBC = white blood cells; NEU = neutrophils; BAND = immature neutrophils aka. band cells; LYM = lymphocytes; MONO = monocytes; EOSIN = eosinophils; BASO = basophils.

[2]The following diet names have been assigned to a respective experimental diet groups: *Control*: soy protein concentrate + no supplemented ISF, fed to both uninfected and PRRSV-infected control pigs; *Control + ISF*: soy protein concentrate + supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM*: ETSBM + no supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM + ISF*: ETSBM + supplemented ISF, fed to PRRSV-infected pigs only.

Table B.5. Effects of dietary soy isoflavones level and porcine reproductive and respiratory virus (PRRSV) infection on serum cytokine concentrations (pg/mL) in weanling pigs[1]

	Uninfected	PRRSV-infected					*P*-value
Item, pg/mL	Control[2]	Control[2]	Control + ISF[2]	ETSBM[2]	ETSBM + ISF[2]	SEM	Treatment
TNF-α							
0 DPI	128	33.2[†]	38.7[†]	39.6	50.0[†]	38.4	0.017
3 DPI	72.7	89.7	120	85.1	104	24.8	0.584
6 DPI	58.7	75.1	140	59.9	73.4	31.0	0.077
14 DPI	52.2	152	113	187	353	52.1	0.158
IFN-α							
0 DPI	18.6	101	202	16.4	134	112	0.642
3 DPI	19.3	1,132	1,483	1,643[†]	2,156[†]	431	0.024
6 DPI	142	501	428	529	396	170	0.401
14 DPI	5.31	44.6	17.1	21.6	50.2	48.0	0.511

[†]Difference ($P < 0.05$) between uninfected control and respective infected group by Dunnett's adjustment when overall treatment effect was present.
[1]Values represent least square means of 10 to 12 pigs. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI = day post-inoculation; ISF = isoflavones; TNF-α = tumor necrosis factor-α; IFN-α = interferon-α; BDL = below detectable limits.
[2]The following diet names have been assigned to a respective experimental diet groups: *Control*: soy protein concentrate + no supplemented ISF, fed to both uninfected and PRRSV-infected control pigs; *Control + ISF*: soy protein concentrate + supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM*: ETSBM + no supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM + ISF*: ETSBM + supplemented ISF, fed to PRRSV-infected pigs only.

Table B.6. Effects of dietary soy isoflavones level and porcine reproductive and respiratory virus (PRRSV) infection on peripheral blood T cell immunophenotypes of weanling pigs[1]

	Uninfected	PRRSV-infected					*P*-value
Item	Control[2]	Control[2]	Control + ISF[2]	ETSBM[2]	ETSBM + ISF[2]	SEM	Treatment
T-Cell, % (CD3+)[3]	55.30	53.21	53.01	53.89	59.02	7.29	0.665
Helper T-Cell, % (CD3+CD4+)[4]	22.03	20.18	23.09	18.03	26.41	2.08	0.075
Cytotoxic T-Cell, % (CD3+CD8+)[4]	12.44	22.78†	22.43†	22.71†	19.20	3.04	0.004
Dual-Positive T-Cell, % (CD3+CD4+CD8+)[4]	4.30	6.66†	6.32†	6.15	5.13	1.26	0.038
Dual-Positive T-Cell Expressing IFN-γ, % (CD3+CD4+CD8+IFNγ+)[4]	90.10	79.72†	79.25†	77.94†	83.41	4.05	0.022
Dual-Positive T-Cell Expressing IFN-γ MFI (CD3+CD4+CD8+IFNγ+)[5]	764.1	501.0	515.4	490.6	577.3	99.0	0.001
CD4:CD8 Ratio	2.15	1.06	1.17	0.918	1.53	0.18	< 0.0001

†Difference ($P < 0.05$) between uninfected control and respective infected group by Dunnett's adjustment when overall treatment effect was present.

[1]Values represent least square means of 10 to 12 pigs with collection of blood occurring at 12 DPI. All pigs received allotted treatment diet starting -7 DPI. Abbreviations: DPI = day post-inoculation; ISF = isoflavones.

[2]The following diet names have been assigned to a respective experimental diet groups: *Control*: soy protein concentrate + no supplemented ISF, fed to both uninfected and PRRSV-infected control pigs; *Control + ISF*: soy protein concentrate + supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM*: ETSBM + no supplemented ISF, fed to PRRSV-infected pigs only; *ETSBM + ISF*: ETSBM + supplemented ISF, fed to PRRSV-infected pigs only.

[3]Percent of total lymphocytes that are positive for cell-surface marker CD3.

[4]Percent of CD3-positive lymphocytes that are also positive for cell-surface markers CD4, CD8, CD4/CD8, or CD4/CD8 and intercellular IFN-*γ*

[5]Median fluorescence intensity (MFI) is a measure of the fluorescence intensity in a fluorescence channel being measured. It provides an alternative measurement to percent positive for comparison of cell populations between individuals. It is less sensitive to outliers, which is important for very small cell populations like the dual-positive T-cells.